IN VITRO MODULATION OF MENISCUS BIOSYNTHESIS: A BASIS FOR UNDERSTANDING CELLULAR RESPONSE TO PHYSIOLOGICALLY RELEVANT STIMULI

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IN VITRO MODULATION OF MENISCUS BIOSYNTHESIS: A BASIS FOR UNDERSTANDING CELLULAR RESPONSE TO PHYSIOLOGICALLY RELEVANT STIMULI

Approved by:

Dr. Marc E. Levenston, Chair
School of Mechanical Engineering
Georgia Institute of Technology

Dr. Lawrence J. Bonassar
Department of Bioengineering &
Mechanical and Aerospace Engineering
Cornell University

Dr. Robert E. Guldberg
School of Mechanical Engineering
Georgia Institute of Technology

Dr. William J. Koros
School of Chemical & Biomolecular
Engineering
Georgia Institute of Technology

Dr. Christopher S. Lynch
School of Mechanical Engineering
Georgia Institute of Technology

Date Approved: July 18, 2005

To Mom, Dad, and Robyn

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LIST OF ABBREVIATIONS

2-D	two-dimensional
3-D	three-dimensional
ACA	ε-aminocaproic acid
ACL	anterior cruciate ligament
ANOVA	analysis of variance
bFGF	fibroblast growth factor-basic
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DMEM	dulbecco's modified eagle's medium
DMMB	1,9-dimethylmethylene blue
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IGF-I	insulin-like growth factor-I
IL-1α	interleukin-1 alpha
$MgCl_2$	magnesium chloride
NEAA	non-essential amino acids
O.D.	optical density
PBS	phosphate buffered saline
pDAB	p-dimethylaminobenzaldehyde
PDGF-AB	platelet derived growth factor-AB
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
sGAG	sulfated glycosaminoglycan
SIS	small intestinal submucosa
TGF-β1	transforming growth factor-beta 1

SUMMARY

The meniscus is a soft, fibrocartilaginous tissue critical for the maintenance of normal knee biomechanics, providing shock absorbance and overall joint lubrication and stability. The adult tissue is highly avascular with a poor autonomous repair capacity in response to injury. Despite the estimated 850,000 arthroscopic surgeries performed per year to repair torn menisci and the increasing evidence showing a high incidence of meniscal degeneration during very early stages of osteoarthritis, little is currently known of the responses of meniscal fibrochondrocytes to physiological stimuli. Therefore, this work explored the responses of meniscal fibrochondrocytes to exogenous biomechanical and biochemical stimuli in an effort to better understand the sensitivity of these cells in their native tissue matrix as well as in a 3-D scaffold environment.

Using the immature bovine model, the changes in biosynthesis of fibrochondrocytes in tissue explants and in an agarose scaffold due to unconfined oscillatory compression were explored. This biomechanical stimulus, previously identified to stimulate matrix production of chondrocytes of articular cartilage, stimulated total protein synthesis in both culture environments. In contrast, the synthesis of proteoglycans, matrix components important in mechanical stiffness and hydration of the tissue, was not affected by these compression protocols. However, the use of a biochemical stimulus in the form of anabolic cytokines significantly enhanced both protein and proteoglycan synthesis as a function of culture environment as well as type of cytokine used. The superposition of oscillatory compression in addition to the use of these potent biochemical stimulators, insulin-like growth factor-I or transforming growth

factor-beta 1, did not further enhance matrix synthesis of fibrochondrocytes in agarose culture, suggesting an insensitivity of the fibrochondrocytes to biomechanical stimuli during early stages of matrix maturation within the agarose scaffold. As a combination of biomechanical and biochemical stimuli are responsible for directing the development, maintenance, and repair of the tissue, these findings aid in understanding fibrocartilage maintenance through studying responses in a tissue explant model. Additionally, studying agarose scaffolds aid in the understanding fibrocartilage development and deposition of a *de novo* matrix.

CHAPTER 1

INTRODUCTION

1.1 MOTIVATION

The meniscus is a soft, fibrocartilaginous tissue that is critical for the maintenance of normal knee biomechanics. The adult tissue is highly avascular with a poor autonomous repair capacity in response to injury[1-3]. Tears of the meniscus are the predominant form of injury to the soft tissue and can result in the need for surgical intervention or meniscectomy to remove the tear and in severe cases the whole meniscus[3]. The American Academy of Orthopaedic Surgeons has estimated that approximately 850,000 arthroscopic surgeries are performed a year to repair or partially remove the meniscus. Even with surgical intervention, meniscal injury leads to an alteration of normal knee biomechanics with increased loading on the underlying articular cartilage and subsequent development of osteoarthritis[4]. Despite the seemingly important role of the meniscus in maintenance of a healthy knee, little is currently know of the cellular responses of native meniscal fibrochondrocytes to physiologically relevant stimuli. Understanding the behavior of these cells in response to physiologically relevant biomechanical and biochemical stimuli is central to understanding normal tissue maintenance. Additionally, characterizing a baseline response of fibrochondrocytes provides a datum for comparison of normal behavior to changes that occur during pathogenesis as well as during engineered regeneration when these cells are placed in a non-native matrix.

1.2 RESEARCH OBJECTIVES

The overall goal of this thesis is to elucidate the effects of biomechanical and biochemical external stimuli that are components of the physiological environment on meniscal fibrochondrocytes in native explant culture and in agarose gel culture. Initially, each of the stimuli will be explored separately to characterize their individual effects on fibrochondrocytes. In the *in vivo* environment of the knee joint, both stimuli affect the development, maintenance, and repair of the meniscus[5,6]. This work aims to understand the biosynthetic responses of fibrochondrocytes to the delivery of polypeptides in a normal homeostatic environment that includes physiological mechanical stimulation.

1.2.1 Specific Aims

There were three hypotheses and three associated specific aims to address the biomechanical and biochemical effects on fibrochondrocytes. To aid in understanding the tissue model used in this work, a preliminary characterization of the immature bovine meniscus was performed. Based on studies of human and animal menisci of varying ages, the meniscus has a complex structure and composition with a heterogeneous distribution of matrix and cells. The preliminary work in this thesis focused on identifying distributions of matrix components in the immature bovine meniscus quantitatively via biochemical analysis and qualitatively via immunofluorescent staining. Fibrochondrocytes from different regions of the menisci were also examined for innate differences in gene expression, comparative behaviors when seeded into a 3-D scaffold, and relative changes in biosynthetic responses to exogenous stimuli in tissue explants.

The results from this work provided justification for tissue and cell isolation procedures of subsequent studies in the thesis and aided in the interpretation of results.

Hypothesis 1: Exogenous mechanical stimuli will affect matrix synthesis of meniscal fibrochondrocytes in native tissue explants and in agarose gels. Static compression will inhibit matrix synthesis, and oscillatory compression will stimulate matrix synthesis.

Specific Aim 1: To determine the effects of physiologically relevant mechanical loading on the biosynthesis of meniscal fibrochondrocytes in native tissue explants and in agarose gels.

The effects of mechanical compression on articular chondrocytes in tissue explants and 3-D scaffolds have been extensively characterized. The current studies examined effects of similar stimuli on the biosynthesis of fibrochondrocytes in their native tissue matrix as well as in agarose hydrogel culture. For meniscus tissue explants, changes in biosynthesis under graded levels of static compression up to 50% were measured and compared to those seen in articular cartilage explants. Similarly, both tissues were subject to oscillatory compression at 1.0 Hz and were analyzed for changes in biosynthesis. Analogous static and oscillatory compression studies were performed for fibrochondrocytes and chondrocytes seeded in agarose gels. Biosynthetic behavior of fibrochondrocytes in agarose gel culture has not been characterized. Therefore, these studies explored the biosynthesis of fibrochondrocytes during early stages of matrix deposition in 3-D agarose scaffolds, potentially aiding in the understanding of potential repair and tissue engineering strategies.

Hypothesis 2: Exogenous biochemical stimuli, specifically anabolic cytokines that are known to stimulate articular chondrocyte biosynthesis will stimulate meniscal fibrochondrocyte matrix biosynthesis.

Specific Aim 2: To determine the effects of individual anabolic cytokines on the biosynthesis of meniscal fibrochondrocytes in native tissue explants and in agarose gels.

In these studies, the effects on fibrochondrocyte biosynthesis of four anabolic cytokines previously shown to modulate chondrocyte matrix biosynthesis alone[7-13] or in concert with the application of mechanical stimuli[14-17] were examined. The cytokines included: fibroblast growth factor-basic (bFGF), insulin-like growth factor-I (IGF-I), platelet derived growth factor-AB (PDGF-AB), or transforming growth factor-beta 1 (TGF-β1). Studying the effects of cytokines on fibrochondrocytes in either tissue explants or agarose gel culture may aid in understanding the specific roles of cytokines in matrix development, maintenance, and repair. Individual effects of several anabolic cytokines, also referred to as growth factors, on the biosynthesis and gene expression of fibrochondrocytes were studied. The first set of studies examined the individual effects on fibrochondrocytes in tissue explants of one of four growth factors. The effects on fibrochondrocyte biosynthesis were examined over a range of concentrations of the chosen anabolic cytokines (dose-response). The second set of studies explored the effects of a single concentration on explants of each anabolic cytokine over a period of two weeks (time-course). Finally, the effects of TGF-β1 or IGF-I at a single concentration on the matrix synthesis and gene expression of fibrochondrocytes and chondrocytes seeded in agarose gels over a period of 14 days were studied. These studies

provided a direct comparison between fibrochondrocytes in their native matrix and in a 3-D scaffold as well as a comparison with chondrocyte behavior in the same 3-D scaffold subject to the identical biochemical environment. Additionally, changes in mRNA expression of typical "chondrocytic" gene expression were determined.

Hypothesis 3: Combining exogenous biomechanical and biochemical stimuli will have a synergistic effect on matrix biosynthesis. Specifically, the superposition of compression on cytokine supplementation will induce changes in matrix biosynthesis that differ from the additive effects of each stimulus applied individually.

Specific Aim 3: To determine the effects of the combination of mechanical loading and cytokine supplementation on the biosynthesis of meniscal fibrochondrocytes in native tissue explants and agarose gels.

The *in vivo* environment of the meniscus contains a milieu of cytokines as well as physiological loading. To simulate this complex environment, these studies used the same stimuli presented in the previous aims to determine the combined effects on matrix synthesis and gene expression. It has been suggested that depending on the combination of stimuli present, effects may be additive or greater than additive, acting in a synergistic manner[15,16]. In the first studies, tissue explants were placed under graded levels of static compression while supplemented with a single level of one of the four growth factors. The second set of studies was performed on fibrochondrocytes and chondrocytes in agarose gel culture. Following a week of preculture in the presence or absence of either TGF-β1 or IGF-I, gels were subject to either graded levels of static compression or an

intermittent oscillatory compression protocol. Thus, interactions between typically stimulatory supplementation with growth factors and either inhibitory (static) or stimulatory (dynamic) biomechanical stimuli were explored.

1.3 Significance and Contributions

The studies described in this thesis aid in understanding the response of native fibrochondrocytes to stimuli that are present in their *in vivo* environment. Biosynthetic responses to both biochemical and biomechanical stimuli were measured, individually and in combinations of both stimuli. Control conditions were included in the study designs in order to give an indication of basal activity of the fibrochondrocytes in different matrices. Increases in biosynthesis over basal activity due to external cues in an *in vitro* setting provided insight into the cellular capacity for response to stimuli that are components of the physiological environment. In addition to native explant cultures, agarose gel studies addressed the response of fibrochondrocytes when seeded in a 3-D scaffold environment, as currently seen in many tissue engineering applications. These studies allowed for the determination of differences in responses to the same stimuli when the cells initially lack interactions with native matrix components. The maturation of the matrix in response to the exogenous stimuli provided implications for meniscal tissue engineering strategies. In both explant and agarose gel studies, comparable chondrocyte groups (in articular cartilage explants or agarose gels) were subject to the identical stimuli. Different responses between these related cell types have implications for repair strategies. As a therapeutic strategy, such as gene therapy or directed growth

factor delivery, may target a single tissue in the knee joint, it is important to also note its effects on the neighboring tissues that share a common synovial environment.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1 MENISCUS STRUCTURE

The menisci are fibrocartilaginous organs in the knee joint that are important in maintaining normal knee biomechanics. Sitting between the ends of the femur and tibia (Figure 1), the pair is firmly attached to the tibial plateau via anterior and posterior fibrous tissue attachments and is responsible for transmission of 50 to 70% of the load within a single joint[18]. In addition to load transmission, the menisci are also recognized as shock absorbers and provide stability and lubrication to the joint[19]. At birth the meniscus is organ fully vascularized. However with aging, the penetration of vascular supply becomes restricted to the outer third of the organ. These vessels come from the perimeniscal capillary plexus that is distributed throughout the joint capsule soft tissue at the peripheral edge of both menisci[20]. This leaves the inner two-thirds of the organ without a vasculature[2,3].

The meniscus is about 70% water[19]. On a dry weight basis, the major extracellular matrix component is the fibrous collagen type I, accounting for about 70% of the matrix[19]. Collagen type I is a ubiquitous protein found in many connective tissues in the body. The protein structure is a molecule of 300 nm in length composed of three coiled subunits (two α1(I) chains and one α2(I) chain) that form a triple helix (Figure 2). The collagen type I molecules align themselves laterally in microfibrils followed by end to end aggregation into fibrils[21]. Fibrillar collagen has been identified to impart mechanical tensile integrity to the meniscus[19]. Collagen type II, the major type of

collagen found in articular cartilage, is present primarily within the inner region of the meniscus and its quantity may be related to increases in loading with animal maturation[20]. Also, collagen type VI is also present and has been identified as an interfibrillar component, aiding in fibrillar organization[22]. Additionally, collagen types III and V are also recognized as matrix proteins[22-24].

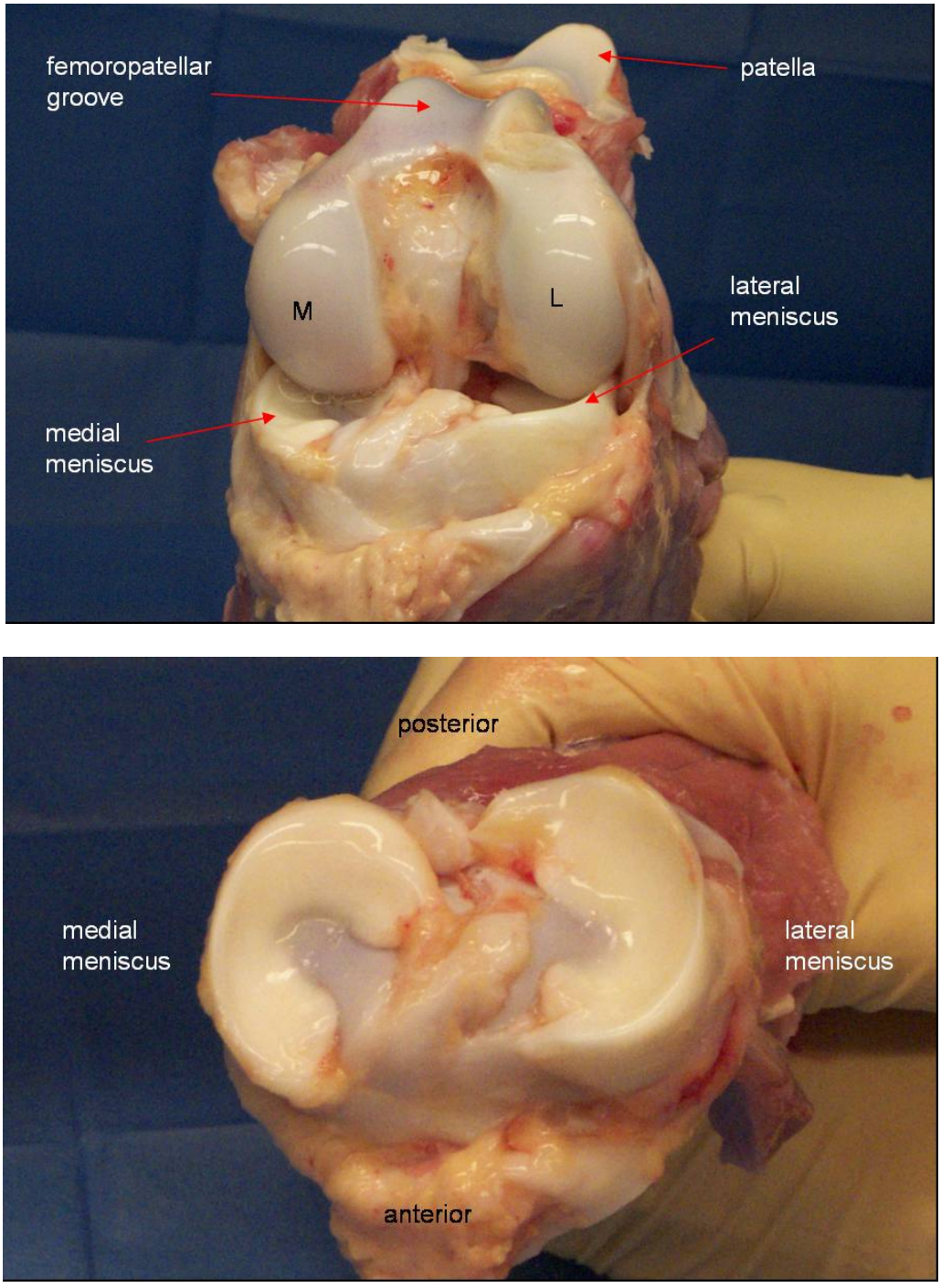

Figure 1: Immature bovine stifle joint. Top photograph shows stifle joint in full flexion after dissection of the intact joint capsule and resection of the patellar tendon. M indicates the medial femoral condyle. L indicates the lateral femoral condyle. Bottom photograph is a caudal view of the tibial plateau with intact medial and lateral menisci.

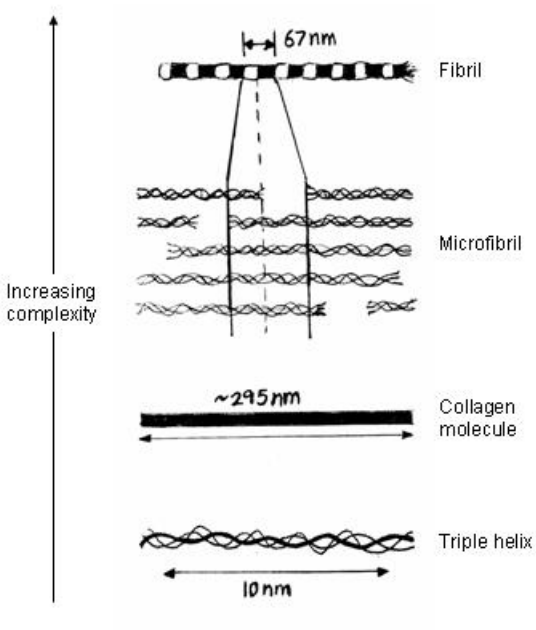

Figure 2: Structural hierarchy of fibrillar collagen. Increasing complexity beginning from the two α1(I) and one α2(I) subunits that form a triple helical collagen molecule. Fibril spacing shown schematically corresponds to molecule spacing within the fibril arrangement. Figure after Orgel *et al.*[25].

Proteoglycans account for 1-3% of the extracellular matrix on a dry weight basis[23,26-28]. Proteoglycans are glycoproteins, consisting of glycosaminoglycans that are covalently linked to a core protein. Glycosaminoglycans are long, negatively charged polysaccharides containing a repeating disaccharide unit. The main proteoglycans found in the meniscus are of two populations: large chondroitin sulfate proteoglycans and smaller dermatan sulfate proteoglycans such as biglycan and decorin. Aggrecan (>2x10^6 kDa, Figure 3), a large aggregating proteoglycan, is the major proteoglycan in the adult bovine meniscus and articular cartilage and contains roughly 100 chondroitin sulfate chains plus keratan sulfate chains in a lesser quantity[20]. These keratan and chondroitin sulfate chains covalently bind to the aggrecan core protein. Multiple aggrecan molecules

can attach to a hyaluronan core in a complex with link protein creating a proteoglycan aggregate that is highly immobilized in the collagen matrix (Figure 4). The sulfate (SO_3^-) and carboxyl (COO^-) groups on the glycosaminoglycan chains create repulsion forces as well as entrain positive counterions producing net electroneutrality. The intratissue ion concentration increases, giving rise to increased fluid pressure (Donnan osmotic pressure) as well as increased ability to resist compressive loading[19].

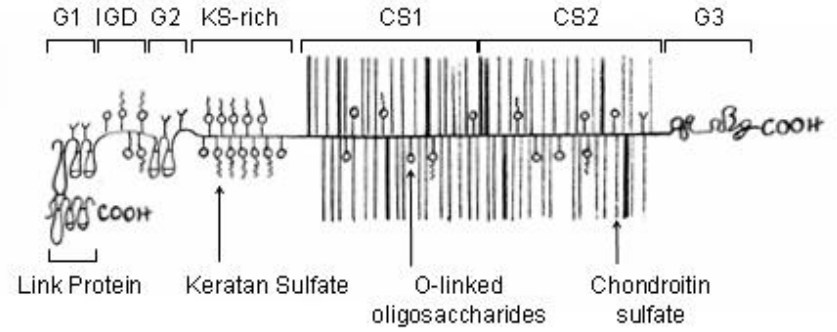

Figure 3: Structure of an aggrecan molecule. G1, G2, and G3 are globular domains. IGD is the interglobular domain between G1 and G2. The KS-rich region contains keratan sulfate. CS1 and CS2 are chondroitin sulfate rich portions of the aggrecan core protein. Link protein forms a complex with the G1 domain aiding in attachment to hyaluronan (see Figure 4). Figure after *GlycoForum (www.glycoforum.gr.jp).*

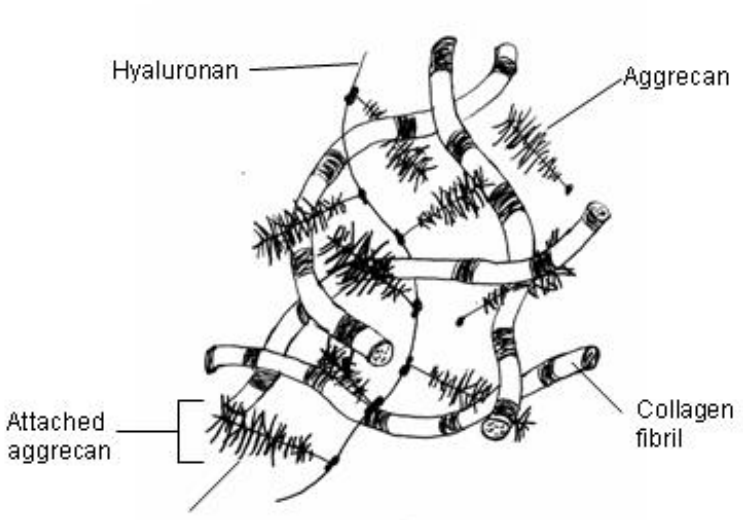

Figure 4: Interactions of extracellular matrix components. Aggrecan proteoglycans attached to a hyaluronan backbone are entangled within collagen fibrils. Figure after Mow and Ratcliffe[29].

These extracellular matrix components are arranged in a highly ordered ultrastructure suited well for the tissue function. Bundles of collagen fibers, mainly type I, are arranged circumferentially in order to resist the tensile hoop stresses developed in the tissue during loading[30]. With this organization, the midsubstance of the meniscus exhibits an anisotropic tensile behavior with the modulus in the circumferential direction ranging from two times to an order of magnitude greater than the modulus in the radial direction[31]. There are also randomly dispersed, radial collagen II fibers believed to "tie" the circumferential bundles together[26], and more recently have been shown to be not just interspersed, but continuous, lining the outer surface of the fiber bundles in a sheath-like manner[32]. Petersen and Tillmann have identified three distinct layers of collagen orientations in adult human menisci using scanning electron microscopy[33]. Layer 1 (Figure 5) or the "superficial network" contains a very thin surface meshwork of fibrils.

This surface lines the femoral and tibial surface of the meniscus. Layer 2 or the "lamellar layer" contains a randomly oriented layer of collagen fibrils and lies immediately below the superficial network. Only at the outermost edge of the meniscus, these fibrils take on a radial orientation. Finally, layer 3 or the "central main layer" contains larger bundles of collagen fibrils that are arranged circumferentially.

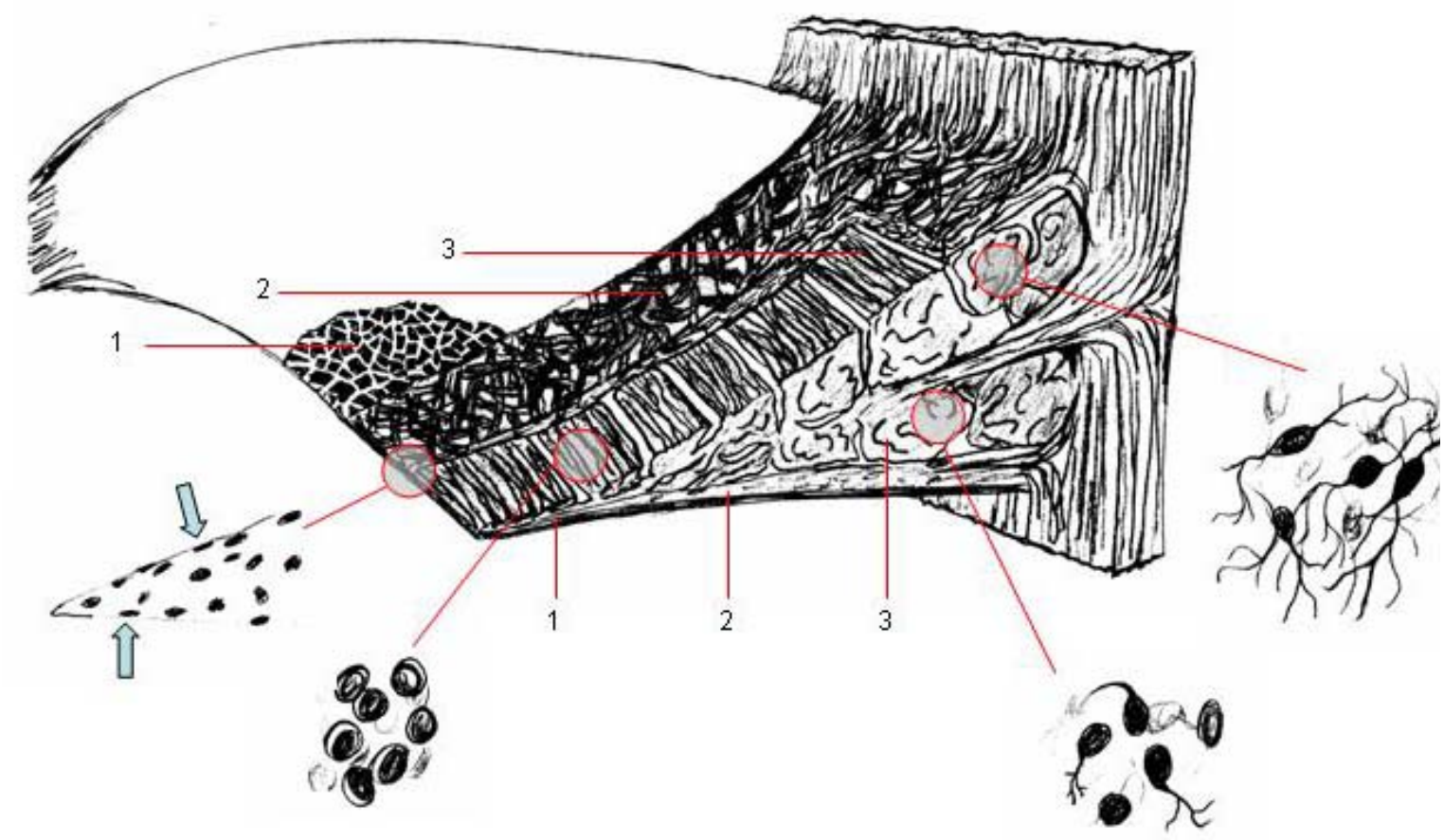

Figure 5: Schematic of collagen fiber orientation in the adult human meniscus based upon scanning electron micrographs. Layer 1 points to the thin meshwork of fibers coating the tibial and femoral surfaces. Layer 2 points to the lamellar layer with mainly random fiber orientation. Level 3 points to the central main layer containing highly oriented collagen fiber bundles. Figure after Petersen and Tillman[33]. Outset sketches show lapine fibrochondrocyte morphology based upon vimentin staining of thick cross-sections. Inset figures after Hellio Le Graverand *et al.*[34].

The proteoglycans are dispersed throughout the extracellular matrix with evidence of local variations in components and concentrations[28]. Within the porcine meniscus, the inner third is reported to have 8% glycosaminoglycan content, mainly chondroitin sulfate, on a dry weight basis, which is greater than the 2% content in the outer third. The increased content of proteoglycans in the inner portion of the meniscus is well suited to resist the mainly compressive strains place on that region[20,35]. The next most prevalent glycosaminoglycan, dermatan sulfate, is reported to exist in all regions of the meniscus, with increased contents in the outer region when compared to chondroitin sulfate content.

The cells of the meniscus that are responsible for producing the finely tuned matrix are referred to as meniscal fibrochondrocytes and are named appropriately, illustrating features typical of both fibroblasts and chondrocytes[36]. Webber *et al.* have shown two distinct populations in monolayer culture: one population with a high proliferative propensity and fibroblastic appearance and a second population with increased matrix synthesis rates and a chondrocytic appearance[37]. Together, these populations are responsible for the maintenance of the fibrocartilaginous extracellular matrix. More recently, Hellio Le Graverand *et al.* have identified four cell populations within lapine menisci based on morphology[34]. From left to right in the lower panels of Figure 5, the first fibrochondrocyte population found consisted of fusiform cells that lined the tibial and femoral surfaces at the inner tip of the meniscus. Moving radially past the surface into the inner compressive region, identified as the region most like hyaline cartilage, the fibrochondrocytes were rounded with no cytoskeletal projections, similar to articular chondrocytes of the middle zone. Fibrochondrocytes in the outer "tensile" zone of the meniscus contained two morphological populations. Cells closer to

the interior of the zone contained fibrochondrocytes with large cell bodies and one or two cytoskeletal projections. Fibrochondrocytes in the outer edge of the meniscus had a stellate appearance with multiple filamentous cytoskeletal projections extending in all directions. When this heterogeneous population was placed in fibrin gel culture, Vanderploeg *et al.* have shown the spontaneous generation of these projections under free swelling culture conditions[38].

2.2 VASCULATURE AND REPARATIVE CAPACITY

The vascular supply of the adult meniscus is limited to the outer one-third of the organ (referred to as the "red-zone"). The remaining two-thirds (referred to as "white-zone") has a small propensity for autonomous repair in response to injury, due primarily to its avascularity[1-3,39]. Previous treatment for meniscal tears typically involved total excision of the injured meniscus. More recently, partial meniscectomy has been favored for several reasons, including the fact that a measurable amount of *de novo* matrix develops upon total removal of the damaged tissue. Even though this matrix is not normal meniscal fibrocartilage, having disorganized collagen fibers and a lower content of proteoglycans [40], the newly formed tissue can contribute to the maintenance of joint stability[27].

Alternate strategies have been studied and implemented in order to increase the reparative response of the tissue to injury. Injuries to the outer "red-zone" of the meniscus are likely to heal autonomously due to the access to the vascular supply and the corresponding chemokines and nutrients supplied[3]. Injuries extending into the avascular

zone show a limited healing potential, but again the repair tissue is not normal fibrocartilage but rather a matrix of collagen with very little proteoglycan[41]. Certain growth and differentiation factors are important in the healing and reparative mechanisms of meniscal tissue, specifically in stimulating fibrochondrocytes to produce necessary matrix components[42,43]. Common clinical repair techniques to increase the healing potential of a meniscal tear include debridement and the creation of vascular access channels[44], allowing a conduit to the vascular supply rich in these factors. The use of fibrin to promote meniscal repair is also a widely explored repair topic. Fibrin clots provide an environment containing many of the same factors from the vasculature that can enhance meniscal repair even in the avascular zone[45-47]. Although lacking the growth factors present in fibrin clots, synthetic fibrin glues are effective adhesives to secure the tear while repair occurs. Specifically, the fibrin scaffold appears to promote cellular infiltration and matrix deposition with successful repair in hircine[48] and lapine[49,50] models as well as in human clinical studies.

2.3 Fibrochondrocyte Response to Exogenous Stimuli

There is a strong structure-function relationship within the meniscus and the other soft tissues of the knee joint. These relationships are developed during maturation, creating intricate and heterogeneous matrices. During development, there are a myriad of external cues that direct development of a functional matrix. This work aims at addressing several specific biomechanical and biochemical stimuli that may give insight

into the manner in which the fibrochondrocytes produce and organize matrix components during development, maintenance, and repair.

2.3.1 Responses to Biomechanical Stimuli

Data from our laboratory has explored the effects of oscillatory tension on matrix biosynthesis of fibrochondrocytes seeded in fibrin gels[38]. Identifying the development of tensile stresses in the outer region of the meniscus upon physiological loading, these studies found a significant decrease in matrix synthesis with tensile load for both fibrochondrocytes and articular chondrocytes.

In other laboratories, researchers at Duke University used the adult porcine model subjected to oscillatory compression using a commercially available compression apparatus under load control[51-53]. Their results showed increases in both protein and proteoglycan synthesis (68% and 58%, respectively) relative to uncompressed controls. Differences in results between our laboratories for proteoglycan synthesis could be due to species and age of the menisci used, as well as differences in the application of oscillatory compression. Their study used a load controlled square wave compression protocol, whereas our laboratory used a displacement controlled sinusoidal compression protocol. They have also looked at the relationship of nitric oxide production in response to static and dynamic loading. Nitric oxide, a free radical implicated in the arthritic degeneration of the meniscus[54], is produced at elevated levels in response to mechanical compression[51]. Additionally, a relationship between nitric oxide production and interleukin-1 treatment, as a model for osteoarthritis, was described[52].

Upton *et al.* studied at the effects of static and dynamic compression on gene expression in the adult porcine menisci[53]. This study found that matrix proteins (collagen types I and II) and proteoglycans (decorin only) were regulated on a transcriptional level by mechanical compression. However, other proteoglycans such as aggrecan and biglycan and cytoskeletal components such as vimentin and β-actin were not affected at the transcriptional level by mechanical compression.

In addition, published *in vivo* studies reported the effects of mechanical loads on the biosynthesis of the fibrochondrocytes. Due to the difficulty in prescribing a precise mechanical environment, as well as interspecies differences, much of the data appear to be contradictory in nature. An example is seen in the comparison of menisci of exercised rats[55] versus menisci of exercised chicks[56]. There was an increase in collagen and proteoglycan content with exercise of the rats, however no change in collagen or proteoglycan content with exercise of the chicks. Using an *in vitro* explant model should decrease variability in results due to the ability to highly control the culture environment.

2.3.2 Responses to Biochemical Stimuli

In an effort to understand the effects of specific cytokines on fibrochondrocytes, many studies have examined cells in monolayer and explant culture[37,40,57-61]. The subset of cytokines in this work represents a group of factors commonly implicated in orthopaedic applications as well as results of preliminary studies looking at the efficacy of a wide range of cytokines. The group of four anabolic cytokines, commonly placed under the category of growth factors with broad specificities, includes: fibroblast growth

factor-basic (bFGF), insulin-like growth factor-I (IGF-I), platelet derived growth factor-AB (PDGF-AB), and transforming growth factor-beta 1 (TGF-β1).

The use of bFGF at 10 ng/mL and ascorbate at 40 μg/mL by Webber *et al.* caused fibrochondrocytes cultured in monolayer to proliferate, whereas using only ascorbate shifted the cells towards increases in matrix deposition, specifically increases in sulfated glycosaminoglycans[37]. A mitogenic response was induced by PDGF-AB in fibrochondrocytes in explant culture[60]. Work of Spindler *et al.* also reported a mitogenic response in adult ovine explants when exposed to 200 ng/mL of PDGF-AB[58]. In addition, work by Bhargava *et al.* with IGF-I showed enhanced migration of isolated fibrochondrocytes only from the "red-white zone"[60]. Collier and Ghosh found increased stimulation of proteoglycan production by ovine fibrochondrocytes in monolayer and explant culture with the addition of TGF-β1 over serum-free media and media containing 10% fetal bovine serum, respectively[40]. TGF-β1 was also noted to stimulate cell division in their monolayer cell culture model. Human fibrochondrocytes in monolayer also demonstrated a dose-dependent increase in proteoglycan production with exposure to TGF-β1[59].

2.4 Comparisons to Articular Cartilage

Although the meniscus is considered to be a fibrocartilage versus the hyaline cartilage that the articular chondrocyte produces, there are many similarities in function and structure that make the articular cartilage an appropriate comparison point for meniscal characterization. Additionally, these tissues share a common environment and

are subject to similar biochemical and biomechanical stimuli *in vivo*. Both tissues are substantially hydrated with water contents of around 70% for the meniscus[27] and up to 80% for articular cartilage. The cells are organized in a specific orientation, where both fibrochondrocytes and articular chondrocytes in the superficial tissue layer are aligned parallel to the surface. The cells in the midsubstance of both tissues appear more rounded and sit within pericellular layers identified as chondrons[62] or fibrochondrons[63].

The extracellular matrices of both tissues are highly suited for the load distribution and lubrication demands within the knee joint. Although the primary collagen type in the tissues differ (collagen type I in meniscus and collagen type II in cartilage), the compositional levels are similar. However, there is a large discrepancy between tissues in the quantity and composition of the proteoglycans present. In articular cartilage, proteoglycans composed of large, aggregating, chondroitin sulfate rich proteoglycans account for 10% of the dry weight. In contrast, the proteoglycans of the meniscus only account for 1-3% of the tissue's dry weight, and in addition to the aggregates seen in articular cartilage, smaller, non-aggregating dermatan sulfate proteoglycans make up a noticeable proteoglycan population of fibrocartilage[23,26].

2.4.1 Responses to Biomechanical Stimuli

Articular cartilage is highly characterized in the literature. Articular chondrocytes in explant culture, monolayer culture, and gel culture have been shown to respond to mechanical loads as well as biochemical stimuli. In addition to the static and oscillatory compressive stimuli presented in this work, studies exploring the effects of hydrostatic pressure[64,65] and shear stress[66-68] on chondrocyte matrix synthesis and gene expression are

reported. Studies relevant to the current work include results showing a significant inhibition of protein and proteoglycan synthesis by chondrocytes in response to static compression. Increasing the static compression percentage caused even greater levels of inhibition[69]. Conversely, when chondrocytes in native tissue explants[69-72] or seeded in a gel culture[73-77] are placed under low amplitude oscillatory compression, significant increases in both protein and proteoglycan production are seen. Stimulation of the chondrocytes has been shown to be highly dependent upon dynamic loading frequency, with Kim *et al.* showing a threshold of at least 0.001 Hz in order to see increases in biosynthesis of 3 mm diameter by 1 mm cartilage discs[72]. The transition frequency is thought to depend on the characteristic "gel diffusion time" governing load-driven convective flow, which depends both on the sample material properties and dimensions[72,73,78].

2.4.2 Responses to Biochemical Stimuli

Studies by Morales and Roberts reported a saturation in TGF-β1 stimulated proteoglycan synthesis by immature bovine articular cartilage explants at a concentration of 10 ng/mL[79]. The actions of bFGF on articular chondrocytes were identified as both mitogenic[80,81] and biosynthetic. Osborn *et al.* reported saturation in proteoglycan synthesis at 100-1000 ng/mL for adult articular chondrocytes[80], with significant stimulation of ^{3}H-thymidine incorporation at the highest concentrations of bFGF. Studies on articular cartilage explants reported plateaus in IGF-I stimulation of proteoglycan synthesis from 20-200 ng/mL[7,11,80,82]. This wide range of concentrations was attributed to

differences in immature versus mature tissue[11,80], where mature tissue required lower concentrations of IGF-I for maximal response in proteoglycan production.

Sah *et al.* showed a consistent response in matrix synthesis for adult articular cartilage with bFGF supplementation[11], but reported that only bFGF at a concentration of 3 ng/mL stimulated matrix production by immature bovine cartilage. Concentrations of 30-300 ng/mL bFGF inhibited both matrix production and mitogenic activity of immature chondrocytes and exhibited a catabolic effect with an increase in proteoglycans released from the tissue matrix.

There are also several studies reporting the i*n vivo* concentrations of cytokines present in articular cartilage. Luyten *et al.* estimated 50 ng of IGF-I per gram (wet mass) of bovine articular cartilage[7]. This value was on the same order of magnitude as the concentrations shown to cause a plateau in matrix stimulation of articular chondrocytes[11,80,82]. Schneiderman *et al.* reported a lower concentration of 10 ng total IGF-I per gram of adult human articular cartilage, with nearly 90% of the total amount bound to binding proteins[83]. bFGF was found in articular cartilage at concentrations of 1-50 ng per gram of tissue[11]. Although there is very little information on the quantity of these cytokines in fibrocartilage, Ochi *et al.* identified the expression of several peptides using immunohistochemical staining[84]. Following a meniscal rasping technique that promoted healing of the meniscus within the avascular zone, expression of both TGF-β1 and PDGF had peaks in expression at 7 and 14 days, respectively.

2.5 AGAROSE GEL CULTURE

The majority of the previous work with fibrochondrocytes has been with cells in explant culture or plated down in 2-D monolayer culture. Isolating the cells from the native matrix and placing them in a 3-D culture environment that is free of native matrix molecules provides a method of studying external stimuli in an environment with fewer confounding factors that may make data interpretation more difficult. Information about the development of matrix components can also be probed in this environment. Unlike the monolayer culture, chondrocytes have been shown to maintain a differentiated phenotype in 3-D cultures that support rounded cell morphologies. Additionally, chondrocytes have been shown to be able to redifferentiate to some extent when placed into 3-D culture following dedifferentiation in monolayer culture[85,86].

Agarose gel culture was chosen for the current studies. Agarose is a hydrocolloid that is isolated from agar or agar-bearing marine algae. Its long chain structure is made up of repeating blocks of the units shown in Figure 6. Agarose has been used extensively in studying chondrocyte matrix development in free swelling culture[85,87,88] and subject to biomechanical stimuli[73,74,76,77,89,90]. On a morphological level, Weber *et al.* report that fibrochondrocytes are able to maintain a rounded cell type, producing a surrounding matrix with significant collagen and sulfated proteoglycans by day 28 of culture[57]. Although from 1990, this study appears to be the only published study looking at the behavior of fibrochondrocytes in agarose. In light of the morphological characterization work of Hellio Le Graverand *et al.*, the use of agarose may limit the fibrochondrocyte to behavior that is characteristic of the rounded cells from the inner compressive region of the tissue[34].

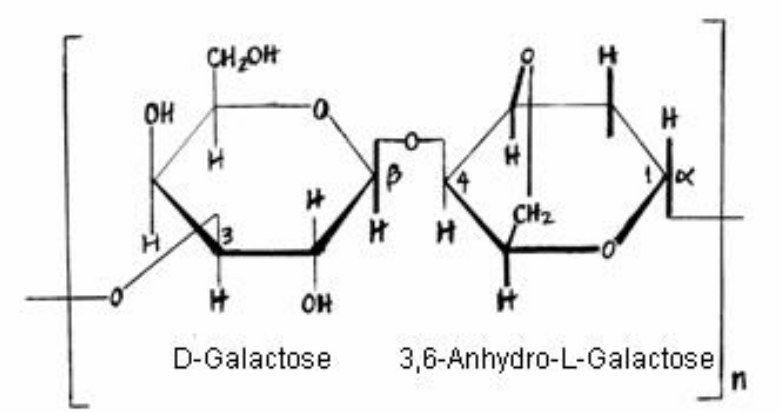

Figure 6: Agarose structure. This linear polymer shown above contains repeating units of D-galactose and 3,6-anhydro-L-galactose forming long chains. Figure after *Sigma-Aldrich Co (www.sigma.com).*

Buschmann *et al.* were the first to report the use of agarose as a scaffold to promote chondrocyte growth of a "mechanically functional cartilage-like matrix"[87]. At the end of a 35 day culture period, chondrocytes in a 2% (w/v) agarose gel had the capacity to develop mechanical properties and matrix component densities that were approximately 25% that of native calf articular cartilage. The same group also included the addition of external mechanical stimuli applied at different time-points of maturation to monitor the effects on newly synthesized matrix components[73]. These studies showed significant differences in matrix synthesis responses that were dependent upon time in culture. Increased amounts of accumulated matrix (i.e., longer time in culture) then led to significant inhibition of matrix synthesis when placed under static compression up to 50%. Additionally, longer culture time induced a variation in matrix synthesis rates across the diameter of the gel in response to low amplitude oscillatory compression. The results of the static compression studies imply that chondrocyte responses to static compression are highly dependent upon cell-matrix interactions and local

physicochemical changes due to decreases in hydration, potentially leading to local changes in fixed charged density and pH levels. In response to dynamic compression, the chondrocytes appear to be highly dependent upon changes in fluid flow, streaming potentials, and cell-matrix interactions, as previously postulated for the response of chondrocytes in native tissue by Kim *et al.*[72]. These issues will be further addressed in the discussion of the current work (see Discussion of Chapters 4 and 6).

2.6 Clinical Issues

2.6.1 Degenerative Disease

Recent clinical diagnostic evidence strongly suggests the early involvement of meniscal degeneration during in the pathogenesis of osteoarthritis. In a survey of a symptomatic population exhibiting chronic knee pain, an early clinical indicator of osteoarthritis, and showing only minor osteoarthritic events based on radiograph findings, greater than 70% of the population had degenerative meniscal lesions based on magnetic resonance imaging[91]. Additionally, a survey of an asymptomatic population of patients ranging from 13 to 76 years old showed meniscal degenerative events in over 50% of the patients, with increasing prevalence with age, also using magnetic resonance imaging[92].

A battery of canine animal ACL transaction models to initiate osteoarthritic degeneration was performed in the 1980s to characterize stages of articular cartilage damage[93-96]. Few studies reported the effects of these models on the menisci of the knee. Meniscal damage in the form of fibrillation and tears following resection was shown by Adams *et al.*[93]. Interestingly the results from Sandy *et al.* showed increased synthesis of

proteoglycans following their ACL transection[94]. Since those studies, there have been very few reports on the coupling of meniscal destruction and development of arthritis. Using a rabbit ACL transection model, Hellio Le Graverand *et al.* have reported changes in meniscal matrix structure, cellular depletion, and gene expression due to resection[97,98]. Their results indicated that specific changes as early as 3 weeks after surgery can provide clues about the localization of the loss of functionality within the damaged tissue.

Recent *in vitro* studies in our laboratory looked at an interleukin-1α model of arthritic degeneration. We found an accelerated release of proteoglycan content with exposure to the catabolic cytokine, with intense staining for aggrecan fragments localized to the compressive inner region of the meniscus. This release preceded the release of other matrix components, specifically collagen, and was markedly earlier than the release of the proteoglycans from similarly treated articular cartilage explants. A strong loss in compressive mechanical properties was also seen with this rapid depletion of matrix components[99]. This inherent susceptibility of the meniscus to arthritic degeneration at earlier stages during disease progression as seen in the clinical studies, supporting our preliminary *in vitro* data, suggests a potentially important chondroprotective role and lends itself to an attractive target for early therapeutic intervention.

2.6.2 Current Reparative Techniques

It is evident that the meniscal matrix is well developed for its functional environment. However, injury to the avascular portion of the meniscus commonly calls for surgical intervention since little or no reparative response is seen. With an incidence rate of 0.7 per 1000 inhabitants a year[100,101], treatment of these tears include suturing or stapling the torn piece, as well as partial or total meniscectomy based on tear severity. Total replacement of the injured meniscus with a devitalized allograft is another option. Although these solutions provide for a short term solution to pain, they do not return the normal knee biomechanics seen in healthy joints. This issue leads to an increased chance for radiologically detected osteoarthritis, seen as soon as 5 years after surgery for patients over 30 years of age[4,102,103]. Therefore, there is an important void to be filled in that none of these repair solutions restore the biomechanical environment provided by the meniscus or biochemical responses by the meniscal cells that are seen in healthy knee joint. In comparison to articular cartilage, little is known about the biosynthetic response of the meniscal fibrochondrocytes to exogenous stimuli. This work focuses on the *in vitro* effects of mechanical and biochemical stimulation on the fibrochondrocytes within tissue explants and agarose gels. Understanding these responses to biologically relevant stimuli will provide the foundation for developing new technologies for biological meniscal repair.

CHAPTER 3

PRELIMINARY CHARACTERIZATION OF THE IMMATURE BOVINE MENISCUS

3.1 INTRODUCTION

This chapter presents preliminary characterization of the immature bovine meniscus. We have chosen the immature bovine (2 – 4 weeks old) as the animal model for the studies throughout the work presented in this thesis. The rationale for using tissue from immature animals was two-fold. First, there is a wide body of literature using immature bovine articular cartilage as a model system. In many of our studies, cartilage controls were used to validate our experimental systems. In later studies, articular cartilage groups were also included as [1] direct comparisons between the meniscus and cartilage (serving as a control group) and [2] actual experimental groups with the data contributing to the current body of literature on articular cartilage. The second reason behind this model was the responsiveness of the tissue to external stimuli. It has been shown that adult articular cartilage, although having increased stiffness and collagen content over that of calf cartilage, exhibits a decreased capacity to respond to oscillatory compressive loading[104] and a reduced responsiveness to different chemokines[82,105].

At the outset of this work, there was no published information about the biosynthetic response of fibrochondrocytes to controlled mechanical stimulation for this animal model or any other animal model. There existed an extensive body of literature that characterized the meniscus composition, vascularity, and material properties for many species of varied ages[20]. However, there had not been a characterization of the

model used in these studies. Therefore, we studied several specific aspects of the immature bovine meniscus to provide preliminary characterization of the model. First, a gross characterization of the matrix components throughout both the medial and lateral menisci was performed. Next a qualitative view of the distribution of matrix components through radial and circumferential cross-sections was obtained via antibody staining and confocal microscopy. In an effort to discover differences in cellular populations that may lead to differences in regional matrix deposition, gene expression from freshly isolated fibrochondrocytes was explored. Additionally, fibrochondrocytes from the different regions of the menisci were seeded into fibrin gels to assess differences in matrix production and changes in gene expression over a two week culture period. Finally, the effects of specific biochemical or biomechanical stimuli on the biosynthesis of tissue explants from the different regions of the menisci were studied.

3.2 MATERIALS AND METHODS

3.2.1 Bovine Meniscal Tissue Composition

Tissue harvest procedure

Both medial and lateral menisci were aseptically isolated from stifle joints of immature (2 – 4 week old) calves within 24 hours of slaughter. Using a #22 scalpel blade, menisci were excised from the joint and placed in phosphate buffered saline (PBS) containing 100 U/L penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Each meniscus was manually divided into *inner* (inner ~2/3 of the radial cross section) and *outer* (outer ~1/3 of the radial cross section) regions that contained similar volumes

of total tissue. This division yielded tissue chunks (n=9-12 per region) from the four distinct regions of the meniscus: *medial inner*, *medial outer*, *lateral inner*, and *lateral outer* (shown as MI, MO, LI, and LO in Figure 7, respectively). Tissue from each region was weighed wet, lyophilized, weighed dry, and digested in 1 mg of Proteinase K per 80 mg of tissue (for a final concentration of 0.2 – 0.4 mg/mL of 100 mM ammonium acetate). Samples were placed in an oven at 60°C overnight.

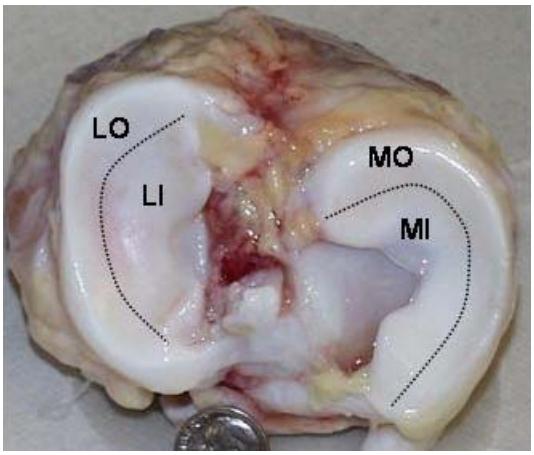

Figure 7: Lateral and medial menisci showing separation into four distinct regions. LO = lateral outer. LI = lateral inner. MO = medial inner. MI = medial inner.

Biochemical quantification

Digested tissue from each region was assayed for general biochemical content. Total DNA content as a measure of total cell content was quantified using the Hoechst 33258 dye assay[106] using calf thymus DNA as a standard read on a Spectra Max Gemini plate reader at an excitation of 365 and emission of 458. Sulfated glycosaminoglycan (sGAG) content was quantified using the colorimetric 1,9-Dimethylmethylene blue

(DMMB) dye binding assay[107] using a chondroitin sulfate (shark cartilage) standard. Total collagen content was quantified using the colorimetric chloramine-T/p-Dimethylaminobenzaldehyde (pDAB) collagen assay[108] using a hydroxyproline standard. Both colorimetric reactions were read on Power Wave 340X-I plate reader at wavelengths of 525 and 557 nm for the sGAG and collagen assays, respectively.

3.2.2 Immunofluorescent imaging

Approximately 3 mm thick "slabs" were taken from lateral and medial menisci. Both circumferential and radial "slabs" were taken from the central third of each meniscus (Figure 8). Samples were immediately placed in 10% formalin for fixation at 4°C for 48-72 hours. They were then rinsed twice for 10 minutes each in PBS. Samples were sliced to a thickness of 50 μm on a Microm HM450 sledge microtome. The sections were then pretreated with 0.25% trypsin in PBS for 30 minutes at 37°C, washed twice with PBS, permeabilized with 1% Triton X-100 for 20 minutes at 37°C, washed twice with PBS, and then blocked with 2% goat serum in PBS for 10 minutes at room temperature. Sections were then incubated overnight at 4°C in one of four primary antibody solutions each containing 2% rabbit serum in PBS plus phalloidin (1:50) and: mouse IgG α-collagen I (1:50), rabbit IgG α-collagen II (1:50), rabbit IgG α-collagen VI (1:50), or rabbit IgG α-aggrecan-G1 (1:50). All primary antibodies were raised against bovine proteins or previously verified to recognize bovine proteins and have negligible cross-reactivity to other collagens or non-collagenous proteins. The following day, samples were washed twice with PBS and blocked with 2% goat serum in PBS for 10 minutes at room temperature. Sections were then incubated for 2-3 hours at 4°C in the

secondary antibody solution containing Hoechst 33258 (1:100 for cell nuclei) and fluorophore-labeled goat α-mouse IgG or goat α-rabbit IgG (1:100) in PBS. Sections were washed in PBS for a final time, mounted on slides, and coverslipped. Images were obtained using confocal microscopy. This work was performed in collaboration with Eric J. Vanderploeg.

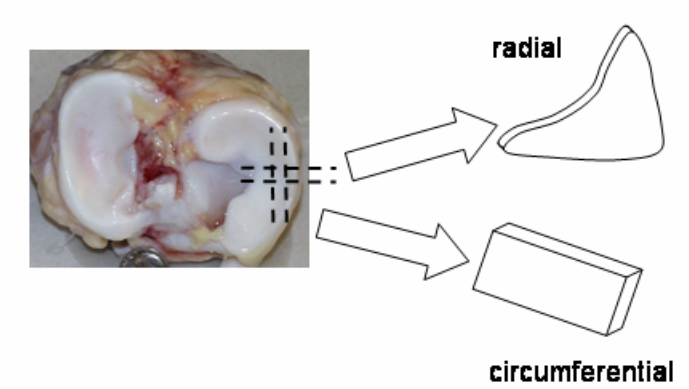

Figure 8: Orientation of the circumferential and radial "slabs" taken for immunofluorescent imaging. Slabs were sliced into 50 μm thick sections.

3.2.3 Gene Expression via Real-Time Quantitative RT-PCR

Cell harvest procedure

As described previously, tissue from distinct regions of the meniscus (*medial inner*, *medial outer*, *lateral inner*, and *lateral outer*) was obtained. The tissue was minced into approximately 2-3 mm^3 chunks, and enzymatically digested using a sequential trypsin-collagenase digestion protocol. Briefly, approximately 3 g aliquots of tissue were placed into T-75 flasks with vented caps allowing for air exchange. Trypsin

solution at 0.025% diluted in PBS was added to each flask (10 mL/flask). T-75 flasks were then placed upright in the refrigerator for 30 minutes. Excess trypsin solution not absorbed into the tissue was aspirated and flasks were placed in a 37°C/5% CO_2 Thermo Electron Corporation incubator for 60 minutes. During this incubation, a 0.4% collagenase solution was prepared in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 50 μg/mL Gentamicin, 50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin, and 100 μg/mL kanamycin sulfate and filtered through a 0.22 μm polyethersulfone filter. For every gram of tissue, 10 mL of the collagenase solution was added. All flasks were then placed on their sides and stacked onto an orbital shaker plate. The shaker plate was placed in the incubator and was set at 150-200 rpm to gently agitate the tissue digests. Upon complete digestion of the matrix components (approximately 38-40 hours in collagenase), the cells were isolated from the digest solution by filtration through a 74 μm mesh. Following sequential rinsing steps using warmed PBS to wash away the remaining enzyme, cells were resuspended in DMEM. Total cell yield as well as cell viability was determined using a ViCell cell counter.

RNA isolation

It should be noted that the current study used fibrochondrocytes isolated using the 2 day collagenase digestion protocol. A more widely acceptable protocol for RNA isolation is to immediately flash freeze the tissue chunks and then pulverize the tissue, isolating RNA from the powder. Due to the fibrous nature of the meniscus, pulverizing the tissue was difficult, and subsequent RNA isolation yielded a very low and impure isolate (since the current study Upton *et al.* have published an optimized protocol for RNA isolation from the meniscus[53]). Therefore, this study used regional cells that were

isolated and digested identically in a 2 day digestion protocol, consistent with the treatment of the cells seeded in fibrin and agarose gels for all presented studies in this thesis.

Following digestion, cell aliquots of 4 x 10^6 cells were pelleted by centrifugation in RNase/DNase free 1.5 mL conicals. Cells were lysed and RNA stabilized with the addition of 350 μL of RLT lysis buffer with 10 μL/mL of β-mercaptoethanol from the Qiagen RNeasy Kit. Samples were mixed well by pipetting and stored at -80°C for subsequent RNA isolation. At the time of isolation, samples were thawed for 10 minutes at 42°C. Lysate was homogenized by centrifugation through a Qiashredder column at 10,000 g for 2 minutes. An equal volume of molecular biology grade 70% ethanol was added to the homogenate and mixed via pipetting. Samples were applied to a Qiagen RNeasy column as per the RNeasy protocol. Briefly, the RNA was bound to the Qiagen RNeasy H-Bind column through centrifugation and was further purified by a series of rinsing steps. RNA was eluted from the column using 60 μL of nuclease free water. The yield of the purified RNA isolate was quantified on a Shimadzu UV-1601 Spectrophotometer by reading a diluted sample (1:50) of the eluted RNA at O.D. 260 for total RNA and O.D. 280 nm for total protein. The concentration of RNA (μg/μL) was calculated from the O.D. 260 by: O.D. 260 * 43 (RNA extinction coefficient)* 50 (dilution factor) /1000 (conversion to μL). The ratio of O.D. 260/O.D. 280 indicated purity of the sample, noting that samples had a ratio greater than 1.5, indicating the high purity of the extracted RNA.

Reverse transcription

RNA was reverse transcribed into cDNA using a Promega Reverse Transcription Kit. Following the protocol from the kit, aliquots of 5 μg of RNA were taken, resuspended in nuclease free water and incubated with 0.5 μg oligo dT primer at 70°C for 10 minutes to remove secondary structures. Next samples were transferred to room temperature for 2 minutes. A master reaction mixture containing 5 mM $MgCl_2$, 1 X reverse transcription buffer, 5 mM dNTPs, Rnasin ribonuclease inhibitor, and 15 units of AMV reverse transcriptase was added. Reverse transcription occurred through incubation at 42°C for 60 minutes, followed by incubations at 95°C for 5 minutes, and on ice for 5 minutes. Each sample was then brought to a final volume of 50 μL of nuclease free water.

Real-time quantitative RT-PCR

Performing real-time quantitative RT-PCR using an ABI Prism 7700 Sequence Detector System, gene expression for the cartilaginous markers of collagen II and aggrecan was determined. In addition, gene expression for the more fibrocartilaginous markers of collagen type I and the smaller proteoglycans of decorin and biglycan was also determined. These bovine primers were developed in our laboratory based on GenBank sequences (Table 1). cDNA (1 μL) was mixed with 49 μL of reaction buffer containing SYBR green I dye, AmpliTaq Gold DNA polymerase, dNTPs, and 0.25 μM of the target primer in a single well. All samples were run in duplicate with a standard dilution series of the appropriate target amplicon and concentration. The 96-well plate was cycled through 40 cycles of a two-step amplification process: incubation at 95°C for 15 seconds to separate strands followed by incubation at 60°C for 1 minute to amplify

DNA. Using the ABI Prism software, threshold intensity and baseline subtraction values were set and the cycle number of amplification crossing the threshold was recorded. Sample expression levels were determined using the cycle values of the standard dilution series.

Table 1: Primer sequences for real-time RT-PCR for bovine collagen type II, aggrecan, collagen type I, decorin, and biglycan.

Gene*	Primer Sequence 5'-3'	Amplicon
Collagen Type II X02420		83 bp
Forward	GCA TTG CCT ACC TGG ACG AA	
Reverse	CGT TGG AGC CCT GGA TGA	
Aggrecan NM_173981		82 bp
Forward	CCT CAG GGT TTC CTG ACA TTA	
Reverse	TAA GCT CAG TCA CGC CAG ATA	
Collagen Type I AB008683		107 bp
Forward	AAG AAC CCA GCT CGC ACA TG	
Reverse	GGT TAG GGT CAA TCC AGT AGT AAC CA	
Decorin NM_173906		119 bp
Forward	ACT GAA GGA ATT GCC AGA GAA	
Reverse	CTA CGA CGA TCA TCT GGT TCA	
Biglycan S82652		85 bp
Forward	GGT CCT CGT GAA CAA CAA GAT	
Reverse	GGA TCT CAC ACA GGT GGT TCT	

* Entries below the gene represent the GenBank accession number.

3.2.4 Fibrin Gels

Freshly isolated meniscal fibrochondrocytes from each region were seeded at a density of 5 x 10^6 cells/mL into fibrin gels. Bovine fibrinogen fraction I was dissolved in DMEM plus 10% FBS and 2 mg/mL ε-aminocaproic acid (ACA) to yield a fibrinogen solution at 50 mg/mL. The cells were then resuspended and mixed thoroughly in this solution. ACA was added directly to the gels as well the feed medium at a concentration of 2 mg/mL to prevent premature degradation of the fibrin matrix due to plasminogen activation. Thrombin at 50 U/mL diluted in 40 mM calcium chloride was added to each well of custom made polycarbonate molds. The cell-fibrinogen solution was added to each well to yield 50 mg/mL fibrin gels of 11 mm diameter x 3 mm height. Polymerization in this model occurs through cleavage of the fibrinopeptides in the E region of fibrinogen monomers by thrombin. The fibrinogen monomers have a "barbell" geometry with the E region located in the center "bar" of the monomer. Upon thrombin cleavage, the E regions can bind to the "bell" regions at the ends of neighboring monomers, aligning end-to-end forming fibrin fibrils.

Fibrin gels were allowed to polymerize for 30 minutes in a 37°C/5% CO_2 incubator. They were then sterilely transferred to serum-supplemented culture medium (DMEM plus 10% FBS, 0.1 mM NEAA, 1.0 mM HEPES, 50 μg/mL gentamicin, 0.25 μg/mL fungizone, and 50 μg/mL ascorbate). Day 0 fibrin gels were immediately taken down following seeding. Medium was changed every other day. Fibrin gels were cultured for up 14 days (n=6 per time-point per region), with gels taken down to probe for sGAG and DNA contents and gene expression on days 2, 4, 7, or 14.

To look at gene expression, an additional purification step was necessary prior to addition of RLT lysis buffer to the samples. Using the TRIspin method of Reno *et al.*[109], the fibrin gels were melted in TRIzol LS Reagent and then homogenized. Chloroform was then added to the homogenate, and phase separation occurred via centrifugation. RNA from the aqueous phase was obtained by an isopropyl alcohol precipitation step. The RNA was pelleted and resuspended in RLT lysis buffer plus β-mercaptoethanol. The RNA isolation protocol stated in the previous section was then followed. Samples (n=3 per time-point per region) were assayed for gene expression of collagen types I and II and aggrecan at days 7 and 14.

3.2.5 Effects of IGF-I on Distinct Regions of the Meniscus

This study examined regional differences in the response of meniscal tissue explants to insulin-like growth factor-I (IGF-I) and contained a slightly different tissue isolation procedure. Menisci were isolated aseptically as previously described. Using a 4 mm diameter biopsy punch, full thickness cores that were oriented perpendicular to the tibial surface were obtained from the four distinct regions of the meniscus: *medial inner*, *medial outer*, *lateral inner*, and *lateral outer* (Figure 9). These cores were sliced to a thickness of 2 mm using a sterilized custom designed cutting block and heavy duty No. 12 razor blades. Superficial surfaces (top and bottom) of the meniscus cores were discarded, as the composition and organization of these regions differ from those of the bulk of the matrix. The tissue disks were soaked in an antibiotic-DMEM solution overnight (DMEM plus 50 μg/mL gentamicin, 50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin, 100 μg/mL kanamycin sulfate, and 0.25 μg/mL

Fungizone) for 18-24 hours and then were pre-cultured for 3 days in basal/serum-free culture medium (DMEM plus 0.1% BSA, 0.1 mM NEAA, 1.0 mM HEPES buffer, 50 μg/mL gentamicin sulfate, 0.25 μg/mL fungizone, 0.4 mM L-proline, and 50 μg/mL ascorbate) to allow for equilibration of all samples. After preculture, meniscus explants from each region (n=6 per region per media condition) were cultured in basal/serum-free medium with or without 200 ng/mL IGF-I for 48 hours. The rationale for using IGF-I in this study will be discussed in Chapter 5. To assess total protein and proteoglycan biosynthesis, 20 μCi/mL of L-5-^{3}H-proline and 10 μCi/mL of ^{35}S-sodium sulfate, respectively, were added to the culture medium during the final 21 hours of culture.

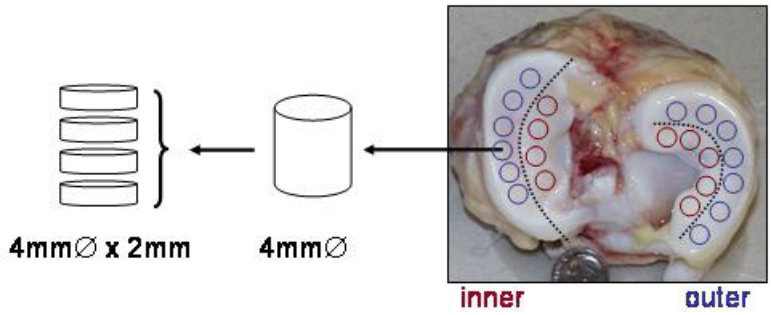

Figure 9: Isolation of meniscus tissue explants from *lateral outer, lateral inner, medial inner,* and *medial outer* regions. Full thickness cores that were oriented perpendicular to the tibial surface were obtained with a 4 mm diameter biopsy punch. The cores were sliced to a thickness of 2 mm, discarding the superficial surfaces (top and bottom).

3.2.6 Effects of Static Compression on Distinct Regions of the Meniscus

This study examined regional differences in the response of meniscal tissue explants to static compression. Meniscus explants, 4 mm diameter by 2 mm thick, from each region were obtained as described above. After the overnight antibiotic-DMEM soak, explants were pre-cultured for 3 days in serum-supplemented culture medium (DMEM plus 10% FBS, 0.1 mM NEAA, 1.0 mM HEPES buffer, 50 μg/mL gentamicin sulfate, 0.25 μg/mL fungizone, 0.4 mM L-proline, and 50 μg/mL ascorbate) to allow for equilibration of all samples. After preculture, meniscus explants from each region (n=6 per region per compression level) were cultured in serum-supplemented media and were either placed under 50% static compression or allowed to free swell in well of a 48-well plate for 21 hours. The application of the 50% compression will be described in detail in Chapter 4. Briefly, explants were loaded with media into a custom designed apparatus that compressed the samples down to 1.0 mm or 50% of the original thickness. To assess total protein and proteoglycan biosynthesis, 20 μCi/mL of L-5-^{3}H-proline and 10 μCi/mL of ^{35}S-sodium sulfate, respectively, were added to the culture medium during the final 21 hours of culture.

3.2.7 Statistical analysis

For all studies data were analyzed with a multi-factor General Linear Model using Minitab Release 12.23. A Tukey's Test for post-hoc analysis was performed if deemed necessary. A value of $p<0.05$ indicated significance. The donor animal was treated as a random variable in all experiments involving tissue from multiple donors.

3.3 RESULTS

3.3.1 Bovine meniscal tissue composition

Measured biochemical composition is presented in Table 2. Tissue from the medial inner region was the most different in composition compared to all other regions studied. It had a higher water content ($p=0.049$) compared to the outer regions. The cell density, as indicated by DNA content per wet mass of tissue, of the middle inner region was the lower than the density in the outer regions ($p<0.0016$). The inner regions had higher sGAG contents on a dry mass basis ($p<0.0005$) than the outer regions. Finally, the medial inner region had a higher collagen content than medial outer explants ($p=0.012$). Pooling data from each meniscus, there were no differences in composition between the two menisci ($p>0.20$).

Table 2: Biochemical composition of the immature bovine meniscus divided into four regions. DNA is expressed per wet mass (μg/mg). sGAG and collagen are expressed per dry mass (μg/mg). [n=9-12 per region, mean ± s.e.m.]

		% Water	DNA (μg/mg)	sGAG (μg/mg)	Collagen (μg/mg)
Medial	Inner	75.3 ± 1.2	0.93 ± 0.07	45.3 ± 9.4	844 ± 55
	Outer	71.8 ± 0.4	1.23 ± 0.03	18.0 ± 2.7	710 ± 10
Lateral	Inner	72.7 ± 1.0	1.07 ± 0.05	37.3 ± 6.6	752 ± 26
	Outer	71.9 ± 0.7	1.07 ± 0.05	22.1 ± 2.0	754 ± 17

3.3.2 Immunofluorescent imaging

The confocal microscopy images illustrated the heterogeneity of the ECM components of the immature bovine meniscus (Figure 10). This heterogeneity occurred

as a distribution in quantity of matrix components as well as organization of these components. The inner regions of the meniscus stained diffusely for collagen types I and II with no detectable organization of the collagen fibrils. Collagen type VI appeared to stain more intensely in the inner region than the other collagens with diffuse matrix staining as well as concentrated pericellular staining. Finally, aggrecan showed the most intense staining in the inner region co-localizing around cells and appearing to provide a connection between cells.

In contrast to the inner regions of the radial "slabs," the outer regions stained for all matrix components in a seemingly organized fashion. The radial "slabs" showed cross sections of large collagen type I bundles co-localized with collagen types II and VI fibers. Similarly organized staining for collagen II was present; however, it was less intense than staining for the other collagens. In general, the collagens appeared to be organized in large fiber bundles composed of smaller fibers in a circumferential orientation. This arrangement was corroborated in the circumferential "slabs" where collagen I stained the heaviest in a wave-like bundle structure. Additionally, staining for DNA showed that cells were located at the intersection of collagen fibers. Aggrecan staining was not as prevalent as in the inner regions however, it also appeared to stain in an organized fashion, similar to that of collagen.

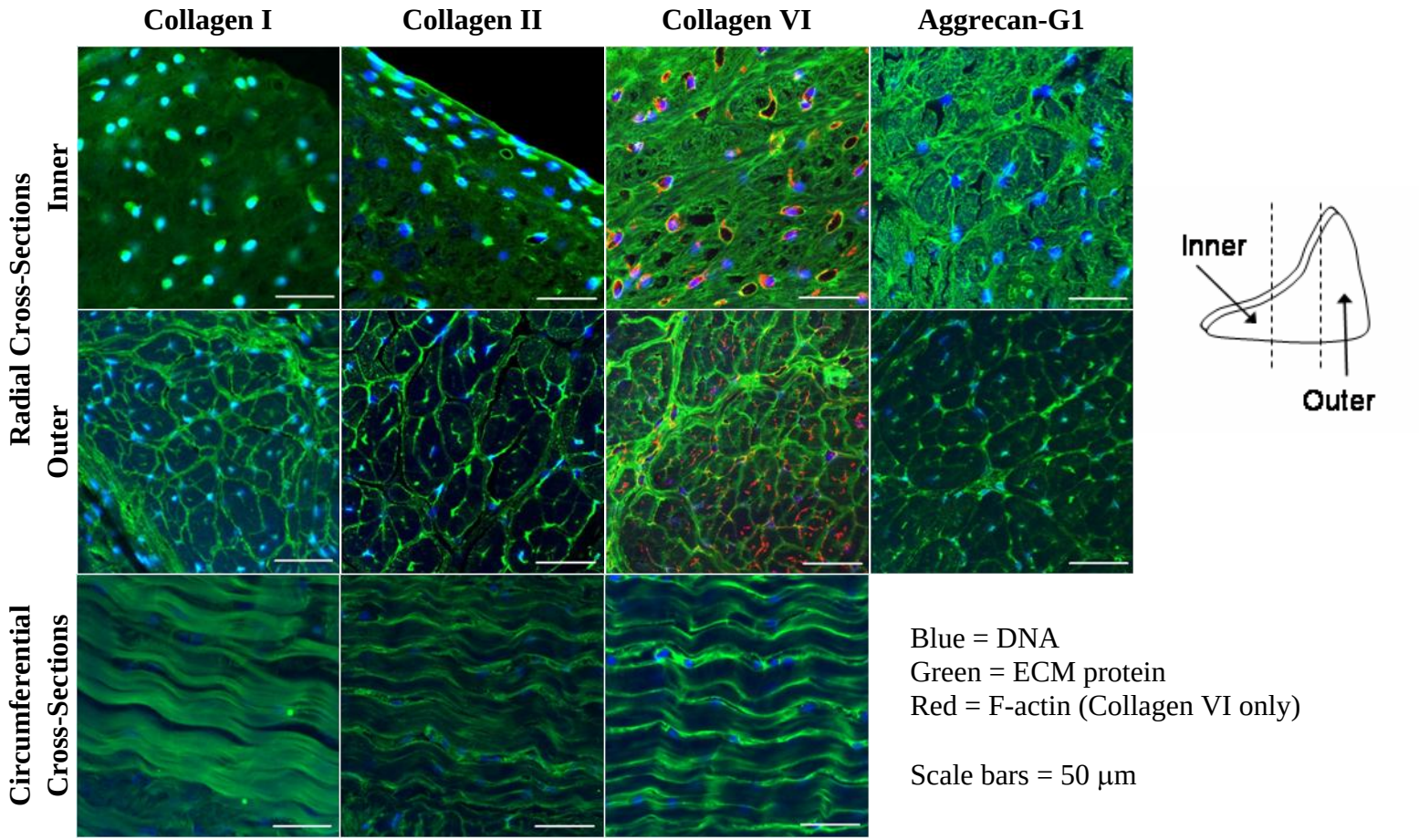

Figure 10: Extracellular matrix structure and distribution in various regions of the meniscus. Images provided by Eric J. Vanderploeg.

3.3.3 Gene Expression

Regional differences of cartilaginous markers were detected in freshly isolated fibrochondrocytes from each distinct region (Figure 11). Collagen type II expression by inner region fibrochondrocytes of both menisci was significantly higher than by the outer region fibrochondrocytes ($p<0.005$). A similar, non-significant trend was seen for aggrecan expression. Comparable levels of collagen type I were constitutively expressed in cells from all regions. Higher expression of decorin was found in outer fibrochondrocytes ($p=0.034$) however, an opposite, non-significant trend was seen for biglycan expression.

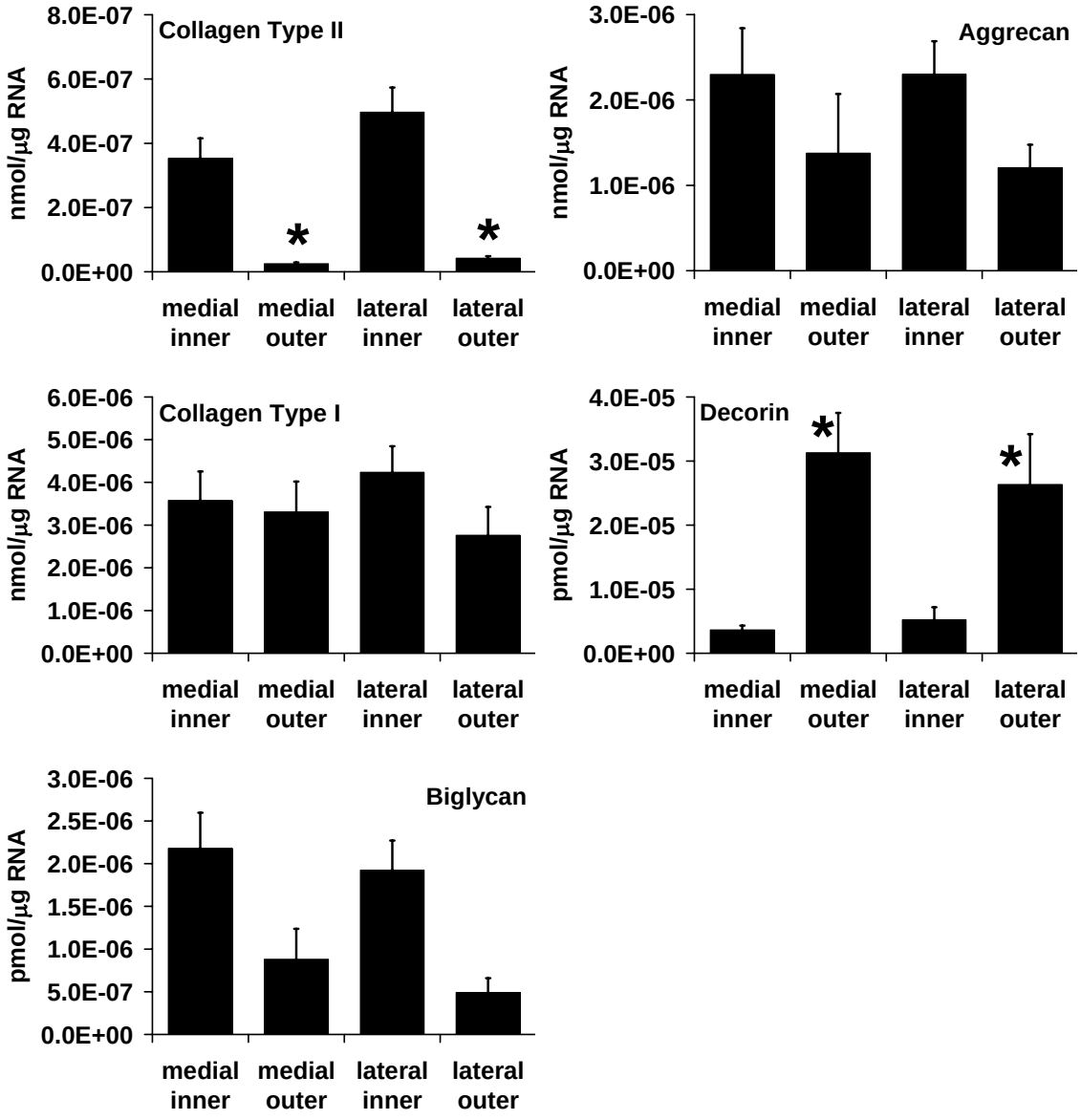

Figure 11: Gene expression of freshly isolated meniscal fibrochondrocytes from each region. Expressed as nmol or pmol of expression normalized to quantity of RNA reverse transcribed (μg).* indicates significant difference from inner regions ($p<0.034$). [n=3 donors per region]

3.3.4 Fibrin Gels

Throughout the time course studied, there were significant differences in matrix accumulation and cellular proliferation among cells from the different regions. The data presented here were analyzed in two different manners. First, a multi-factor General Linear Model was applied to the whole data set with two factors: region of cell source and day of analysis. Second, the data from each time-point were reanalyzed using the single factor of region to compare these effects at a single time point.

Overall, there were significant differences in sGAG accumulation (Figure 12) that depended on the day of analysis ($p<0.001$) as well as the regional cell sources ($p<0.001$). Accumulation of sGAG in the fibrin gels was the greatest for both lateral groups over the medial groups ($p<0.036$). This accumulation occurred rapidly between days 2 and 4 in the culture period with the sGAG content at day 2 being significantly less than the contents measured at any other day ($p<0.0001$). Interestingly, when analyzing the data within an individual day, there was a significant difference in sGAG content among regional cell sources on all days ($p<0.001$) with the exception of day 14 where there was no significant differences in sGAG contents within any of the gels.

The DNA content of the fibrin gels showed significant changes (Figure 12) throughout the culture duration ($p<0.001$) with no significant differences among regional cell sources ($p=0.20$). The data were normalized by DNA content of the respective region's Day 0 gels to account for variation in DNA content due to seeding efficiencies. Analyzing the data on a daily basis showed differences in normalized DNA content with trends that were not similar between days, however by day 14 of culture there were no

significant differences among any cell source in the total DNA content relative to day 0 contents ($p=0.15$).

Gene expression by the fibrochondrocytes seeded in fibrin gels showed similar regional trends among the three genes studied: collagen type II, aggrecan, and collagen type I (Figure 13). For any one of the genes of interest, there was not a significant difference in gene expression among the cells from different regions of the meniscus. There was a significant downregulation of gene expression for collagen type II ($p<0.0001$) and aggrecan ($p=0.0001$) between days 7 and 14. Collagen type I did not show a significant trend with time in culture ($p=0.88$), however due to the small sample size in this preliminary study and large variation in the data, it is difficult to obtain a firm conclusion on the collagen type I expression.

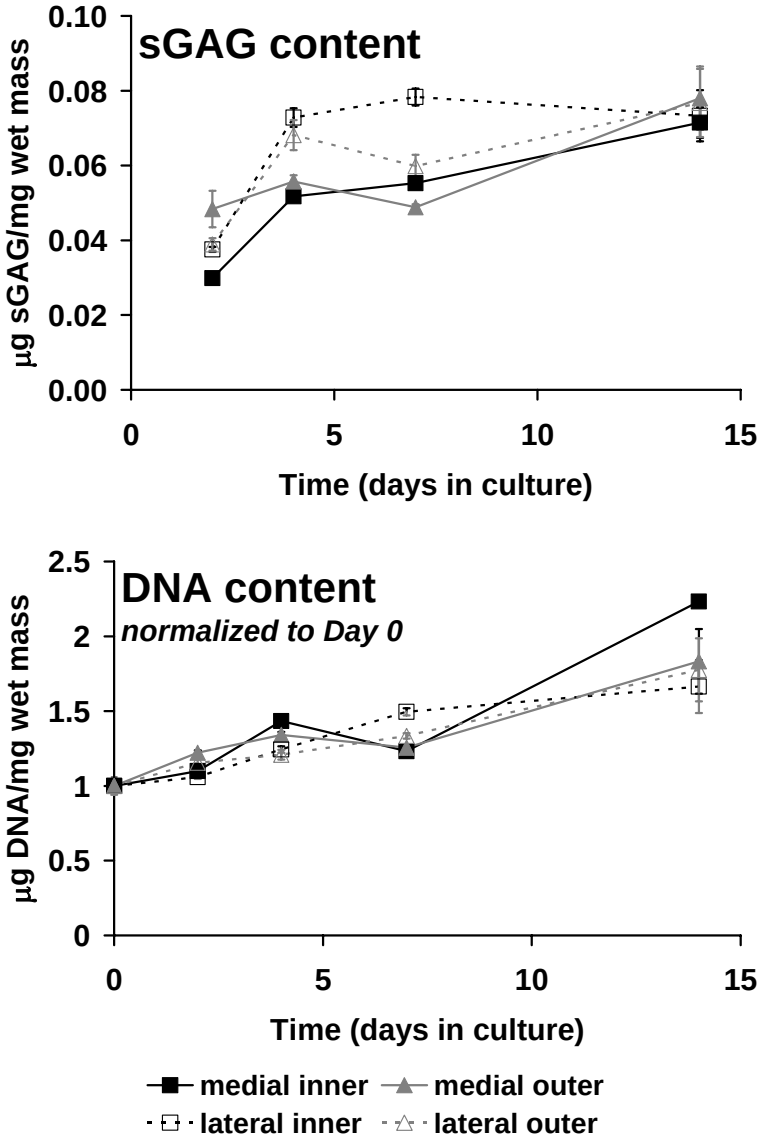

Figure 12: sGAG and DNA contents of fibrin gels seeded with fibrochondrocytes from each region and cultured for up to 14 days. DNA content is normalized to DNA content of Day 0 gels for each individual group. [n=6 per time-point per region]

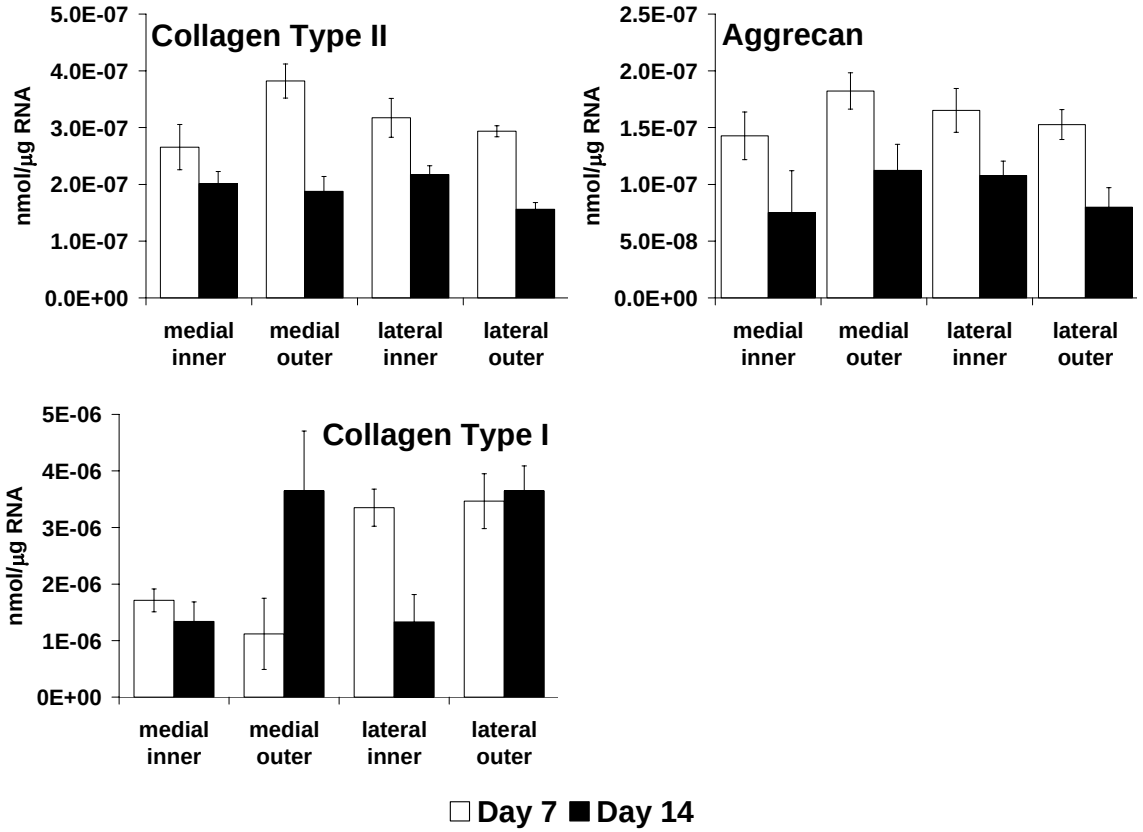

Figure 13: Gene expression in fibrochondrocytes of collagen type II and aggrecan at days 7 and 14. Expressed as nmol of expression normalized to quantity of RNA reverse transcribed (μg). [n=3 per time-point per region]

3.3.5 Effects of IGF-I on Distinct Regions of the Meniscus

For tissue explants from all regions, supplementation with 200 ng/mL IGF-I significantly increased both proline and sulfate incorporation rates over BSA controls ($p<0.003$, Figure 14). Proline incorporation rates were significantly higher in both inner groups compared to only the medial outer group ($p<0.0051$). Lateral inner explants had the highest sulfate incorporation rates over explants from all other regions ($p<0.0009$).

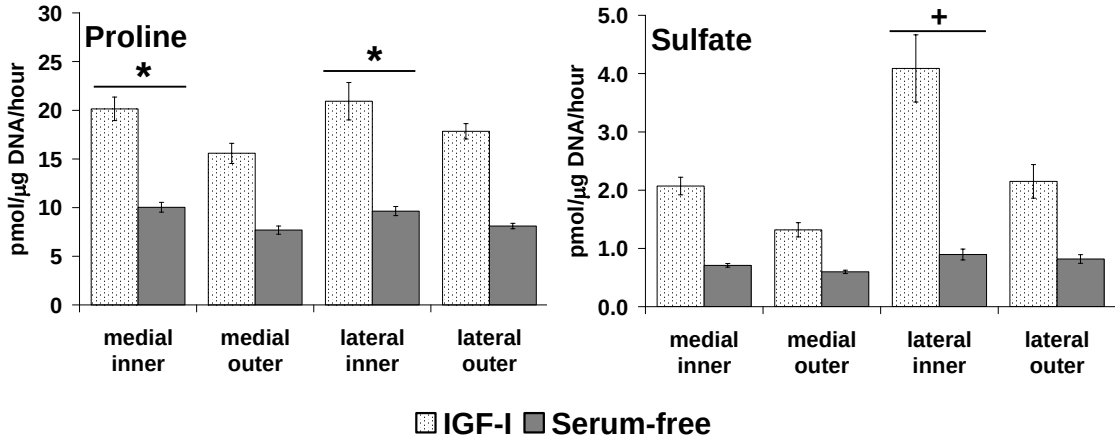

Figure 14: Effects of 200 ng/mL of IGF-I on meniscus tissue explants from different regions. * indicates significant difference from medial outer ($p<0.0051$). + indicates significant difference from all other regions ($p<0.0009$). [n=6 per media condition per region]

3.3.6 Effects of Static Compression on Distinct Regions of the Meniscus

For tissue explants from all regions, application of 50% static compression significantly inhibited proline incorporation rates as compared to free swell controls

(p<0.001, Figure 15) with no significant differences among regional incorporation levels. Sulfate incorporation was not significantly affected by static compression for any region, but explants from the lateral inner region incorporated higher amounts of sulfate compared to all other regions (p<0.0001).

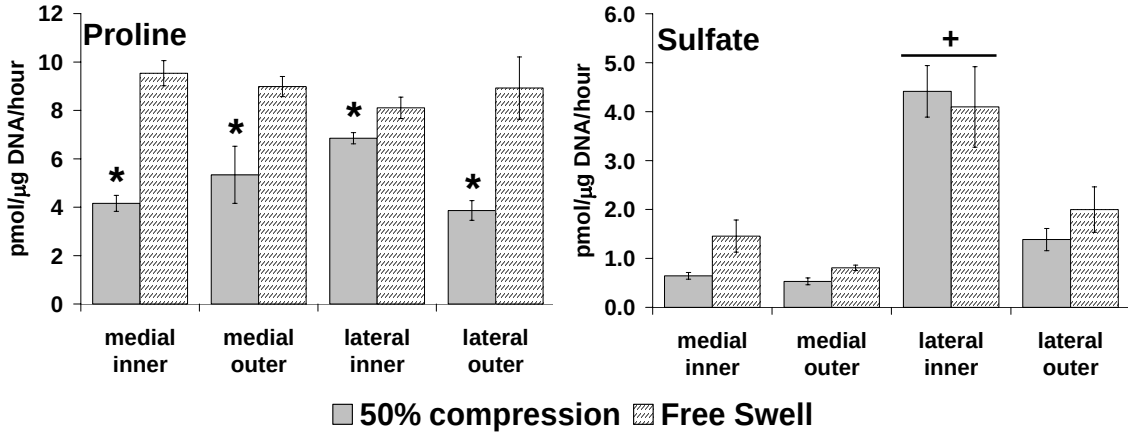

Figure 15: Effects of 50% static compression on meniscus tissue explants from different regions. * indicates significant difference from free swell (p<0.001). + indicates significant difference from all other regions (p<0.0001). [n=6 per compression condition per region]

3.4 DISCUSSION

The characterization presented here provides a baseline for understanding the tissue that is being studied. Global findings included the verification of a heterogeneous extracellular matrix composition as well as inherent differences in gene expression in the different regions of the meniscus. Additionally, transient regional differences were seen when seeded into fibrin gel culture.

Both menisci were separated into inner and outer regions for biochemical analysis and cell isolation. The rationale for this was related to the distribution of matrix components as well as the limited vascular supply that exists in the tissue. Typically, it is assumed that the vascular supply is limited to the outer one-third (side closest to the joint capsule). However, the human newborn meniscus has been found to be entirely vascularized[110] with avascular regions developing within 2 years of life and ultimately reaching 85-90% avascularity in humans[111]. This temporal difference in exposure to vascularity and subsequently the growth and differentiation factors found in the vascular supply can dictate differences in the matrix composition. Although we did not aim to determine the extent of vascularization throughout the cross section of the immature menisci, we did find differences in the quantity of matrix components. Our findings were consistent with characterizations of other species finding increased levels of proteoglycan content in the inner regions. There was likely an increased fixed charged density in the inner regions due to increased localization of proteoglycans, which led to the increase in hydration in that area. Also, it has been hypothesized that this finding across species is an adaptation to loading, as this region undergoes the highest compressive loads *in vivo*[28,112].

The immunofluorescent images presented correlated well with the findings of Kambic and McDevitt in their characterization of the skeletally mature canine menisci[32]. Their work was the first to identify a collagen type II network within the meniscus. Canine radial slab sections were dual labeled for collagen types I and II and showed co-localization of these proteins within the midsubstance of the menisci. Although our studies did not include dual labeling, the work suggests that there was also co-localization

of these proteins in the immature bovine meniscus due to the similar distributions and spacing of the two individual markers. We also found that collagen type VI distribution was suggestive of a third co-localized protein in the fibrocartilage matrix. From the gene expression data, we found that the fibrochondrocytes express genes for both collagen types I and II, and we would expect that these cells would also exhibit expression of collagen type VI, given this matrix distribution and abundance[22].

In the circumferentially oriented slabs, both studies found large oriented collagen type I fiber bundles. Additionally, thinner fibers of collagen type II in the same orientation existed. We found collagen type VI fibers of medium thickness with a greater distance between fibers in this view. These findings showed the diversity of the fibrochondrocytes in their ability to functionally organize a highly oriented matrix composed of multiple collagen types. The relative location of cells at fiber junctions suggested that these cells can interact with the collagen matrix supporting a cell-matrix interaction mechanism for sensing mechanical deformation. Finally, the intense localization of the aggrecan-G1 antibody correlated well with the general biochemistry finding of increased glycosaminoglycan content in the inner regions of the immature bovine meniscus.

The gene expression of the prevalent extracellular matrix components appeared to correlate well with the findings of the immunofluorescent imaging and what has been previously reported for biglycan and decorin contents in the porcine meniscus[112]. Expression of collagen type II and aggrecan, genes typically considered “cartilaginous,” had increased expressions in the inner regions of fibrochondrocytes of both menisci with significantly greater expression of collagen type II only. These levels of expression,

although clearly detectable, were significantly less than the levels that are expressed in chondrocytes of articular cartilage (data not shown). A steady level of the collagen native to fibrocartilage, collagen type I, was expressed by cells from all regions. Taken with the immunofluorescent images of collagen type I, this finding suggests that although the levels of gene expression of this protein are comparable among the fibrochondrocytes from different regions, there is a difference in the amount and extracellular organization (i.e., the inner region containing heavy staining vs. the outer region composed of large directionally organized bundles) of the collagen.

In these studies we did not characterize the quantity or distribution of the smaller proteoglycans in the meniscus. In contrast to the large aggrecan molecules, both biglycan and decorin are significantly smaller with biglycan composed of a protein core and 2 chondroitin sulfate or 2 dermatan sulfate chains and decorin composed of a protein core and 1 chain of either chondroitin sulfate or dermatan sulfate. Trends in gene expression of these leucine rich proteoglycans were opposing, with significantly higher expression of decorin in the outer regions and higher, non-significant expression levels of biglycan in the inner regions. These patterns of expression correlated well with the findings of Scott *et al.* showing highest amounts of biglycan in the inner, compressive region of the meniscus[112]. As the role of biglycan is still undetermined, it has been suggested that it plays a role in the development of connective tissues subject to compressive loading, as higher amounts of biglycan have been found in younger tissue[28]. There was a higher content of decorin in the outer regions of the porcine meniscus. It has been shown that the presence of these proteoglycans can alter the collagen fibril formation and

organization[113] *in vitro*, hence hinting at their importance in organizing the large collagen fiber bundles in the outer regions of the meniscus.

Identifying inherent differences in gene expression of the fibrochondrocytes from the different regions, the last studies looked at the changes of these differences when the fibrochondrocytes were placed in a 3-D scaffold. Fibrin was the scaffold of choice due to the extensive work in our laboratory by Eric J. Vanderploeg in characterizing matrix deposition and biosynthesis of chondrocytes and fibrochondrocytes in response to changes in culture duration and subject to oscillatory tension[38]. At the end of the two week culture period, it appeared that the fibrin gels seeded with fibrochondrocytes from the four distinct regions converged to a similar endpoint. There were no differences in sGAG or DNA contents at the end of the culture period. No differences in expression levels of the three genes of interest (collagen types I and II or aggrecan) were observed by the end of the culture period. Revisiting the experimental setup, an alternative scaffold such as agarose or alginate may have given different results from what was seen in the fibrin environment. The main reason for these potential differences could be cell-scaffold interactions. The fibrochondrocytes in a fibrin scaffold have been shown to take on spread morphology by interacting with the fibrin and forming 3-D projections containing F-actin and vimentin filaments[38]. In contrast, using an inert scaffold that has been shown to support rounded cell morphology, may direct the fibrochondrocytes towards a more chondrocytic phenotype.

The explant data in response to these specific biochemical and biomechanical stimuli showed several differences in the responses of fibrochondrocytes from the distinct regions of the meniscus. The main differences were attributed to increased proline and

sulfate incorporation rates of the lateral inner explants. In general, explants from all regions responded similarly to IGF-I with significant increases in both proline and sulfate incorporation rates compared to the basal media condition. All explants inhibited proline incorporation in response to static compression at 50% with no effects on sulfate incorporation. In general, the responses to the stimuli were the same between the regional explants when compared to paired control conditions. Taken together with the fibrin gel data showing a convergence of the regional cells to a similar endpoint, these data support the use of meniscus tissue explants and fibrochondrocytes from all regions of both menisci. For the rest of the work presented in this thesis, meniscus tissue explants were taken from the middle-outer regions of the menisci (see Figure 4.1 in the following chapter). In all studies, explant locale was recorded at the time of excision, and then explants were randomly assigned to treatment groups giving no apparent effects of source location on the presented data. Additionally for fibrochondrocyte isolation, all meniscus tissue from both menisci was enzymatically digested, pooled, and seeded into agarose gel culture for these studies.

In conclusion, this work has characterized the immature bovine meniscus using several different approaches. The heterogeneous biochemical composition of the menisci did not show any striking differences from what has been reported, with similar spatial distributions, for other species including human[20,114]. Immunofluorescent imaging further supported the distribution of matrix components and also showed spatial variation in the structural organization of these components. Finally, the behavior of fibrochondrocytes from the different regions in tissue explants and fibrin gels responded in similar manners in all culture environments studied. These findings show that despite

the variations in matrix composition, when cultured *in vitro*, the fibrochondrocytes did not show a strong dependence on location of origin in response to the exogenous stimuli explored in this thesis.

CHAPTER 4

DIFFERENTIAL EFFECTS OF PHYSIOLOGICALLY RELEVANT LOADING ON THE BIOSYNTHESIS OF MENISCAL FIBROCHONDROCYTES IN EXPLANTS AND AGAROSE GEL CULTURE

4.1 INTRODUCTION

From the general introduction, it is clear that there is a strong structure-function relationship driven through the developmental process that creates heterogeneous matrices of the soft tissues within the knee. The effects of a broad range of mechanical stimuli on articular chondrocytes in explant culture[66,69-72], monolayer culture[67,68], and gel culture[73-75,77,89,90] have been characterized in the literature. In light of the expansive knowledge on the effects of physiological loading on articular cartilage explants, these studies address similar issues for the meniscus. It is important to begin to understand the effects of biomechanical stimuli on the meniscus, as these cues (along with biochemical cues, studied in Chapter 5) prompt the cells to produce and organize matrix components during development, maintenance, and repair.

Complementing the data obtained for fibrochondrocytes in native explant culture; the following studies also explored the effects of compressive loading on fibrochondrocytes in agarose gel culture. Stripping fibrochondrocytes from their native culture and placing them in a 3-D environment without a matrix template could lead towards an understanding of the development of matrix components around these cells in response to mechanical stimuli. Fibrochondrocytes in these studies were seeded into agarose hydrogels. Unlike the fibrin scaffold system studied in the previous chapter, this uncharged, highly inert matrix allowed for little interaction between the cells and the

hydrogel matrix. Chondrocytes have been shown to encapsulate themselves within the agarose gel by depositing matrix pericellularly at early time-points. After one month in culture, significant amounts of matrix were accumulated with matrix deposition extending and creating an interterritorial matrix[16,73].

Based on what we know about the behavior of articular chondrocytes in native explants culture and agarose gel culture, we hypothesized that meniscal fibrochondrocytes in both of these systems would respond in similar manners to external mechanical stimuli. Understanding the biosynthetic responses of fibrochondrocytes under physiologically relevant loading regimes will aid in the understanding the processes of normal matrix production and maintenance. To measure the biosynthetic response of the cells, the incorporation rates of radiolabeled precursors were studied. Changes in incorporation rates of L-5-^{3}H-proline and ^{35}S-sodium sulfate were used to indicate total protein and proteoglycan production, respectively. These studies explored the effects of physiologically relevant levels of static and oscillatory compression on meniscal fibrochondrocytes in native tissue explants and agarose gel culture. We hypothesized that static compression would inhibit biosynthesis of fibrochondrocytes in either culture system with inhibition of biosynthesis that was dependent upon compression level. We also hypothesized that oscillatory compression would stimulate biosynthesis of fibrochondrocytes in either culture system.

4.2 MATERIALS AND METHODS

4.2.1 Tissue Harvest Procedure

Aseptic techniques as described in Chapter 3 were used to obtain full thickness cores of meniscus explants using a biopsy punch from the middle-outer regions of both menisci (Figure 16). Additionally, full thickness articular cartilage cores were obtained from the femoropatellar grooves and femoral condyles of immature bovine stifle joints. These cores, 4 mm in diameter, were then sliced to a thickness of 1 or 3 mm using the cutting block as described in the previous chapter. The cartilage disks were obtained from the middle zones of the full thickness cores to obtain disks of a homogenous composition and matrix organization. The tissue disks were soaked in an antibiotic-DMEM solution overnight for 18-24 hours and then were pre-cultured for 3 days in serum-supplemented culture medium to allow for equilibration of all samples. Additionally, 0.4 mM of L-proline was added to the all media formulations for the full culture period in which radiolabel incorporation rates were to be assessed.

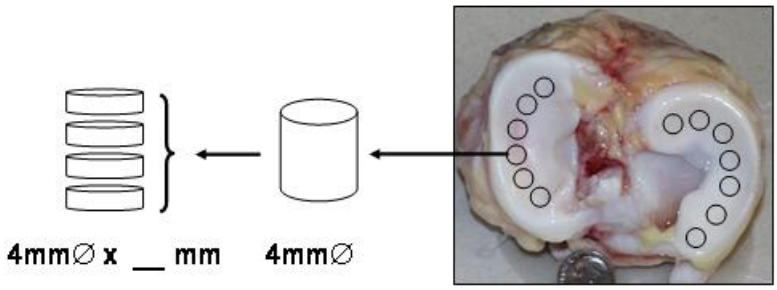

Figure 16: Isolation of meniscus tissue explants from lateral and medial menisci. Full thickness cores that were oriented perpendicular to the tibial surface were obtained from the middle-outer regions with a 4 mm diameter biopsy punch. The cores were sliced to the prescribed thickness, discarding the superficial surfaces (top and bottom).

4.2.2 Cell Harvest Procedure

Chondrocytes and fibrochondrocytes were obtained from enzymatic digestion of the cartilage and meniscus matrices, respectively. The fibrochondrocyte isolation procedure was followed as described in Chapter 3. Chondrocytes were digested using a single stage digestion protocol with 0.2% collagenase alone. Articular cartilage was minced into approximately 2-3 mm^3 chunks. Approximately 3 g aliquots of tissue were placed into T-75 flasks with vented caps allowing for air exchange. For every 1 g of tissue, 10 mL of a 0.2% collagenase solution prepared in the antibiotic-DMEM was added to each flask. All flasks were then placed on their sides and stacked onto an orbital shaker plate. The shaker plate was placed in a 37°C/5% CO_2 incubator and was set at 150-200 rpm to gently agitate the tissue digests. Upon complete digestion of the matrix components (approximately 38-40 hours in collagenase), the cells were isolated from the digest solution by filtration through a 74 μm mesh. Following sequential rinsing steps using warmed PBS to wash away the remaining enzyme, cells were resuspended in DMEM. Total cell yield as well as cell viability was determined using a ViCell cell counter.

Agarose gels

Chondrocytes and fibrochondrocytes were seeded separately into 3% (w/v) agarose gels at a cell density of 5 x 10^6 cells/mL. Low gelling point agarose was reconstituted in calcium and magnesium free PBS. The agarose was fully solubilized and consequently sterilized through a 30 minute liquid cycle in a Steris autoclave. The agarose solution was kept at 42°C to prevent gellation. The cells were resuspended in the molten agarose and dispensed into a mold. This custom designed mold was made of two

glass electrophoresis plates and red FDA rubber. The rubber provided a drip proof seal and an appropriate distance between plates for gel casting. The assembly created a conduit to form an agarose slab of approximately 100 mm x 100 mm x 3 mm. A biopsy punch was used to obtain gels with final dimensions of 6 mm in diameter by 3 mm in thickness. The actual thickness measured with a resistance digital micrometer setup was 2.93 mm with a standard error of 0.025 mm. The agarose gels were placed in 24-well plates, 1 gel/well, with 1 mL of basal/serum-free medium (DMEM plus 0.1% BSA, 0.1 mM NEAA, 1.0 mM HEPES, 50 μg/mL gentamicin, 0.25 μg/mL fungizone, 0.4 mM L-proline and 50 μg/mL ascorbate). Medium was changed every other day. Gels were allowed to preculture for 3 days in basal/serum-free medium.

4.2.3 Static Compression

Tissue explants

Tissue explants were compressed within custom designed polycarbonate static compression chambers (Figure 17). Each chamber base held up to 16 samples in individual wells (15 mm diameter x 10 mm), and samples were compressed between the well bottoms and 8 mm diameter impermeable platens attached to the lid (schematic shown in Figure 17). Three stainless steel spacing blocks and a central annular spacer were used to limit the compression imparted by the platens on the tissue explants. The chamber was placed on an aluminum platform and a nut and washer assembly was used to slowly tighten the chamber lid to the platform/base assembly. After the initial tightening of the chamber lid, appropriate culture media were added to the chamber wells

via the medium ports on the chamber lid. These ports allowed for the aspiration of old medium and addition of fresh medium while maintaining compression of the samples.

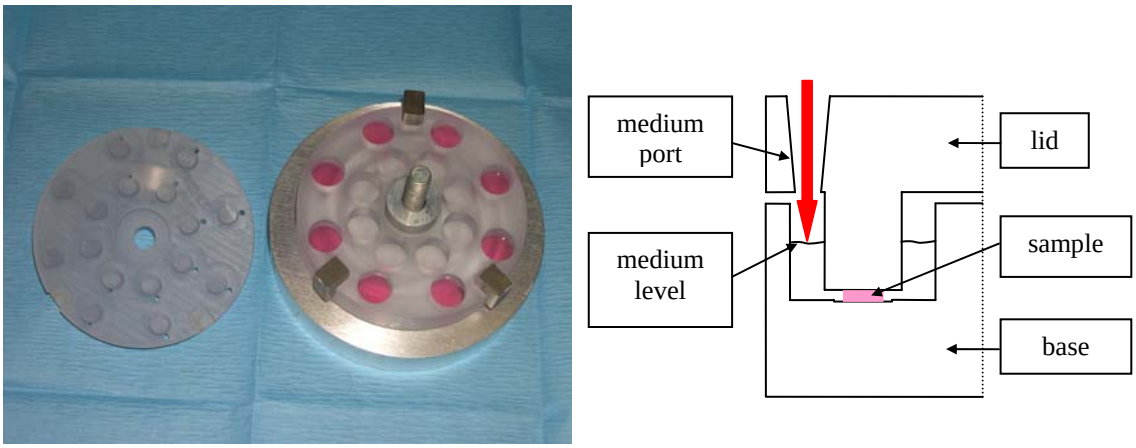

Figure 17: Static compression chamber. Individual samples were placed in isolated wells in the base and compressed between the base and platens on the lid (left). The schematic (right) shows the cross section of a single well containing a sample and feed medium.

Samples were compressed to levels in reference to their originally cut thickness. Tissue samples, cartilage explants in particular, have a tendency to swell upon excision and culture *in vitro*. Samples were compressed to 100%, 75%, or 50% of their stated original cut thicknesses and will hereafter be referred to as 0%, 25%, or 50% compression, respectively. These static compression levels were chosen both to span the range required to produce a consistent inhibitory response and to allow comparison to results in the articular cartilage literature[14,69,71]. Even though the highest levels of compression appear to be non-physiologic, these levels were chosen because they could be seen *in vivo* due to prolonged exposure to loading.

Following the 3 day preculture in serum-supplemented culture medium, cartilage and meniscus tissue explants (4 mm diameter x 1 mm thick, n=8 per compression level per tissue) were statically loaded in radiolabeled medium to assess total protein and proteoglycan biosynthesis using 20 μCi/mL L-5-^{3}H-proline and 10 μCi/mL ^{35}S-sodium sulfate, respectively. The effects of static compression were measured over 21 hours with the addition of the radiolabeled precursors.

Agarose gels

Following the standard 3 day preculture in basal/serum-free culture medium, agarose gels (6 mm diameter x 3 mm thick, n=8 per compression level per cell type) were continued in free-swelling culture for an additional 7 days in basal/serum-free culture medium, allowing extracellular matrix components to accumulate within the gels. On the 7th day (10 days from seeding), the gels were loaded into the static compression chambers in medium supplemented with 10 μCi/mL L-5-^{3}H-proline and 5 μCi/mL ^{35}S-sodium sulfate, and cultured for 21 hours under compression. An additional group compressed to 90% of the original gel thickness (or 10% compression group) was explored in the gel studies. For both tissue and gel studies, a free-swelling group was included. These samples were allowed to culture in a single well of a 24- or 48-well plate and were run in parallel to the statically loaded samples.

At the end of the compression period, the samples were removed from the chambers and washed 4 times for 30 minutes each in PBS supplemented with 0.8 mM sodium sulfate and 1 mM L-proline at 4°C in order to rinse out unincorporated isotopes. Samples were then weighed wet, lyophilized, weighed dry, and digested. Cartilage and meniscus samples were digested as previously described in Chapter 3. A sequential

Proteinase K and agarase digestion was performed on the agarose gels. This protocol allows for the digestion of the extracellular matrix components before full digestion of the agarose. Lyophilized gels were rehydrated in 100 mM ammonium acetate buffer and heated to 100°C in heating blocks for 10 minutes to melt the agarose. The melted gels were then equilibrated in a 60°C oven and Proteinase K was added to the digest solution of each sample at the same concentrations used for the tissue samples. After 24 hours in the oven, samples were again heated to 100°C in heating blocks for 10 minutes to deactivate the Proteinase K. Samples were then equilibrated in a 43°C oven and 2 U of β-agarase were added to the digest solution of each sample. After more than 3 hours in the oven, samples were cooled to room temperature and assayed.

Sample digests were measured for radiolabel incorporation by adding 100 μL of the radioactive digest solution to 2 mL of Ecolume. The prepared samples were then read on a Perkin Elmer Tri-Carb 2900TR liquid scintillation counter. On the day of loading, 1 mL aliquots of fresh radiolabeled media (^{35}S-only and dual-labeled) were stored for use as standards for the radiolabel incorporation quantification. Samples were also assayed for DNA content in order to analyze rates of incorporation on a per cell basis.

4.2.4 Oscillatory Compression

Tissue explants

Articular cartilage and meniscus tissue explants (4 mm diameter x 3 mm thick, n=4 per compression condition per tissue) were placed under oscillatory compression using the device extensively documented in Hunter's[75,115] manuscripts and dissertation[116]. The prescribed compression was a stepped sinusoidal waveform with amplitude of 90 μm

or 3% of the total explant thickness, superimposed upon a 10% compressive offset at a frequency of 1.0 Hz and 0.1 Hz (Figure 18 shows the 1.0 Hz waveform). Control samples were held at the 10% static offset. The tissue explants were placed under oscillatory compression over a 21 hour period with media supplemented with radiolabeled precursors at concentrations of 20 μCi/mL L-5-^{3}H-proline and 10 μCi/mL ^{35}S-sodium sulfate. At the end of the 21 hour loading period, tissue samples were taken down as previously described.

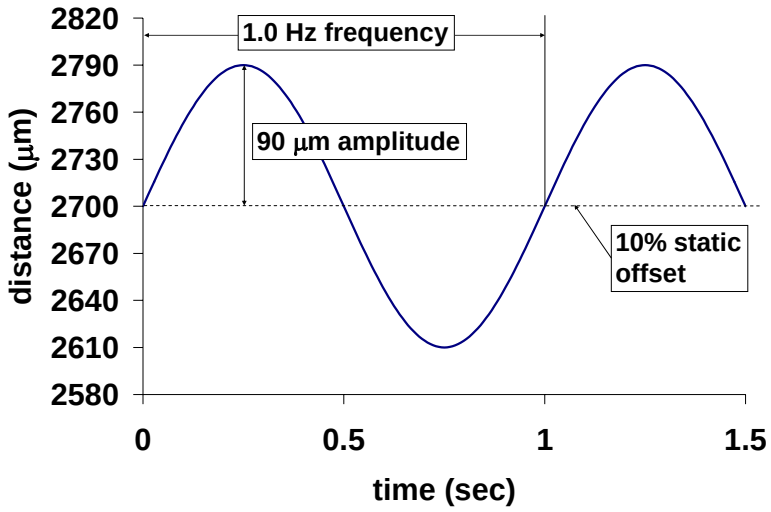

Figure 18: Input waveform for oscillatory compressive loading. The sinusoid has amplitude of 3% and is superimposed upon a 10% static offset. This schematic shows the profile for a 3 mm thick sample.

Agarose gels

Agarose gels (n=8 per compression condition per cell type) were placed under oscillatory compression using a newly developed device that is fully described in Appendix A. Briefly, the entire oscillatory loading system (Figure 19) was designed to fit on a shelf within an incubator. The compression chambers (inset of Figure 19) were made of polysulfone, and a single chamber held up to eight samples. The samples were placed in the individual wells on the bottom part of the assembly (well dimensions of 15.8 mm diameter x 12.7 mm). Steel platens (9.53 mm diameter x 12.7 mm) were attached to the top part of the chamber assembly, providing an impermeable surface to impose the compression. The chambers were designed to have the capability of functioning either in the oscillatory mechanical loading frame or alone to impose static compression. Spacers made of stainless steel were machined to specific heights in order to statically compress the samples to a given height.

The application of oscillatory compression was attained by controlling the motion of a Parker Automation 404XR square rail linear table. The BE231 servo motor was driven by a VIX500AE servo drive, both manufactured by Parker Automation. Using the Galil WSDK Programming Software, the sinusoidal input for table motion was sent to a Galil DMC-2113 servo controller. The translation of motion from the table to the samples was performed using an L-shaped bracket that is made of 6061 aluminum. The tops of the chambers affixed directly to the bracket, while the bottom of the chamber sat stationary on the base plate. The position of the bracket was detected using a 0.1 μm resolution Renishaw linear encoder that was mounted external to the linear table. This linear encoder allowed for feedback control within the system.

The prescribed compression was programmed in the WSDK command language and input as a sinusoidal waveform with amplitude of 30 μm around a 10% static offset at a frequency of 1.0 Hz. As described in the previous Static Compression section, the agarose gels were allowed to preculture for a total of 10 days prior to the application of loading. An intermittent oscillatory compression was applied a total time period of 48 hours using an adapted protocol from Chowdhury *et al.*[77]. Oscillatory compression was applied for 12 hours and followed by 12 hours of static compression at 10% static offset. A second block of compression was immediately performed with another 12 hours of compression followed by 12 hours of static compression at 10% static offset. This protocol was shown to induce the greatest stimulation of proteoglycan synthesis by chondrocytes in agarose gels[77]. Radiolabeled precursors at concentrations of 10 μCi/mL L-5-^{3}H-proline and 5 μCi/mL ^{35}S-sodium sulfate were present in the feed medium throughout the duration of the loading period. At the end of the 48 hour loading period, agarose gels were taken down as previously described.

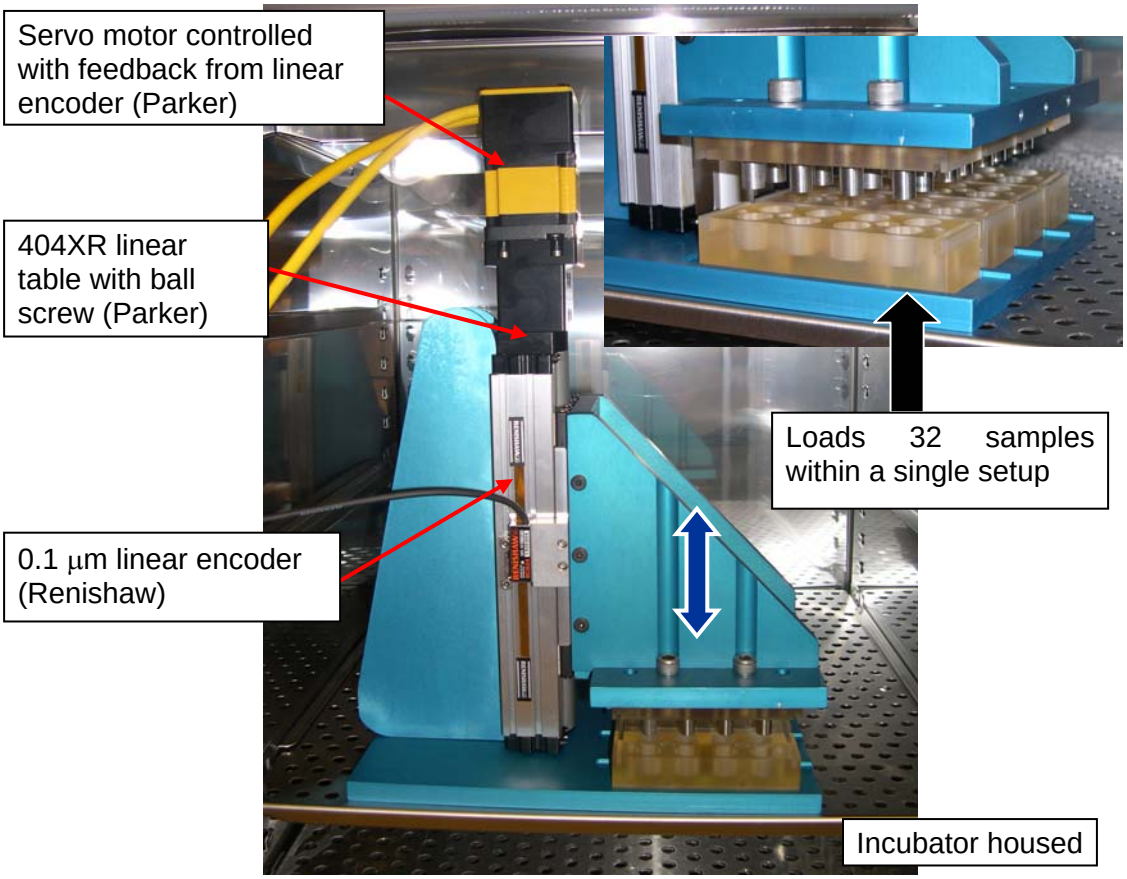

Figure 19: Oscillatory compression loading device. Two identical devices were fabricated and are detailed in Appendix A. The four polysulfone chambers (see inset) hold 8 samples each for a full capacity of 32 samples. Both devices can fit on a single shelf (side by side) within an incubator.

4.3 RESULTS

4.3.1 Static Compression

Tissue explants

Application of static compression over 21 hours significantly affected the biosynthesis of fibrochondrocytes and chondrocytes in native tissue explants. With increasing percentages of static compression over 0% there was an inhibition of both proline and sulfate incorporation rates (Figure 20), indicators of total protein and sulfated glycosaminoglycans, respectively. Levels of 25% compression significantly decreased proline ($p<0.0001$) and sulfate ($p=0.0085$) incorporation rates relative to the free swell controls in both meniscus and cartilage tissues.

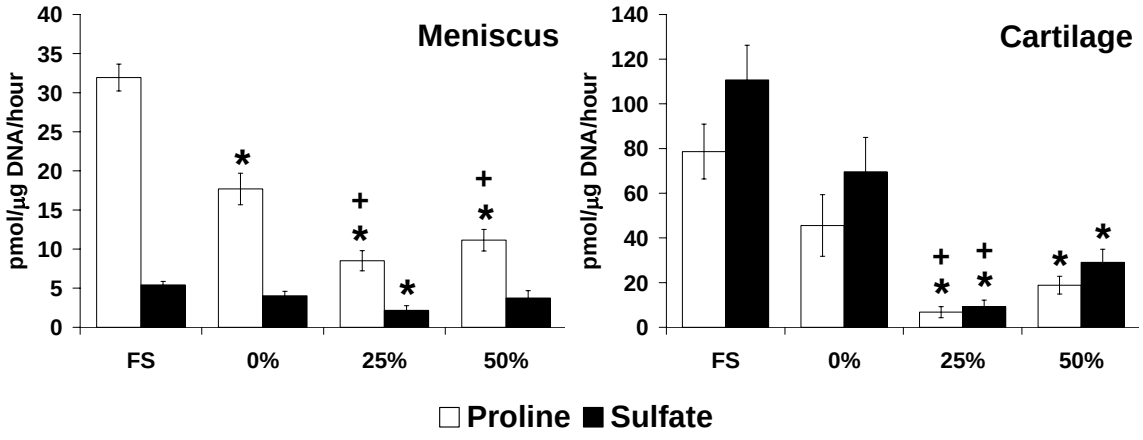

Figure 20: Effects of static compression up to 50% on meniscus and cartilage tissue explants. FS = free-swell group. * indicates significant difference from FS ($p<0.0085$). + indicates significant difference from 0% ($p<0.039$). [1 mm thick, n=8 per compression level per tissue]

Agarose gels

Agarose gels seeded with either chondrocytes or fibrochondrocytes exhibited different responses to static compression over 21 hours. Static compression did not inhibit proline or sulfate incorporation rates compared to free swell controls for either cell type (Figure 21). There were no significant differences in proline or sulfate incorporation rates for fibrochondrocytes in agarose gels under static compression up to 50% compression. There was a trend towards inhibition of fibrochondrocyte biosynthesis for gels held at 50% compression relative to free swell controls, however, this was not significant ($p>0.055$). Interestingly, articular chondrocytes exhibited a non-significant trend towards increasing proline and sulfate incorporation rates for all compression levels except 50% compression, as compared to the free swell controls. Chondrocyte sulfate incorporation was stimulated at 10% compression relative to free swell controls ($p=0.0034$). Compression at 0%, 10%, and 25% induced both proline ($p<0.020$) and sulfate ($p<0.0074$) incorporation rates that were significantly higher than rates of chondrocyte gels held at 0% compression.

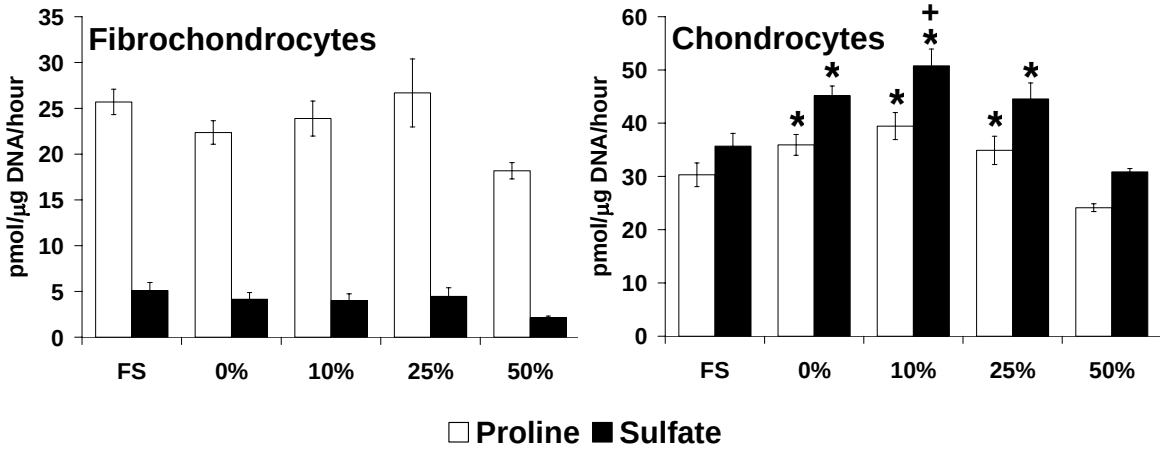

Figure 21: Effects of static compression up to 50% on agarose gels seeded with fibrochondrocytes and chondrocytes. FS = free-swell group. * indicates significant difference from 50% ($p<0.020$). + indicates significant difference from FS ($p=0.0034$). [3 mm thick, n=8 per compression level for fibrochondrocytes n=4 per compression level for articular chondrocytes]

4.3.2 Oscillatory Compression

Tissue explants

Application of oscillatory compression superimposed upon a 10% compressive offset at 1.0 Hz caused a significant increase in proline incorporation rates for both cartilage and meniscus tissue explants ($p=0.014$) as compared to statically compressed controls (Figure 22). This result matched what had previously been shown in the articular cartilage literature. The meniscus behaved in a similar manner with comparable overall levels of proline incorporation rates. Sulfate incorporation rates of cartilage explants subject to 1.0 Hz oscillatory compression also increased significantly over

statically compressed controls (p=0.014). In contrast, there was no effect of oscillatory compression on sulfate incorporation rates in meniscus tissue explants (p>0.71).

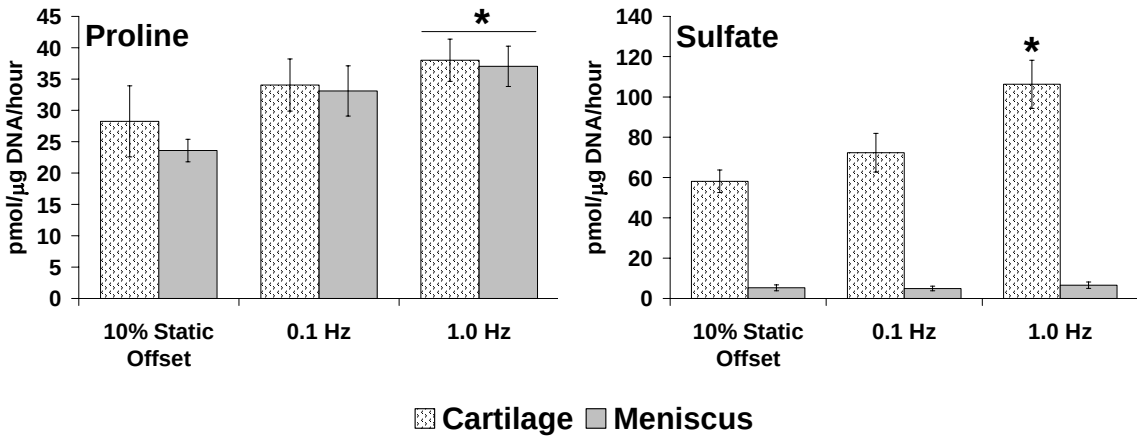

Figure 22: Effects of continuous oscillatory compression (10% static offset ± 3% at 0.1 Hz and 1.0 Hz) on proline and sulfate incorporation of articular cartilage and meniscus tissue explants. * indicates significant difference from 10% static offset (p<0.014). [n=4 per compression condition per tissue]

Agarose gels

Application of oscillatory compression superimposed upon a 10% compressive offset at 1.0 Hz caused different effects on proline and sulfate incorporation rates between the two cell types studied (Figure 23). Oscillatory compression significantly increased both proline (p=0.0079) and sulfate (p=0.038) incorporation rates in fibrochondrocytes over 10% static controls. In contrast, oscillatory compression of agarose gels seeded with chondrocytes did not influence incorporation rates of either proline (p=0.082) or sulfate (p=0.086) in comparison to rates for the 10% static offset

controls. One point to note is that the data presented for the agarose gels represents data for the control medium condition of 0.1% BSA. This condition differs from the control medium condition of 10% FBS for the tissue explants.

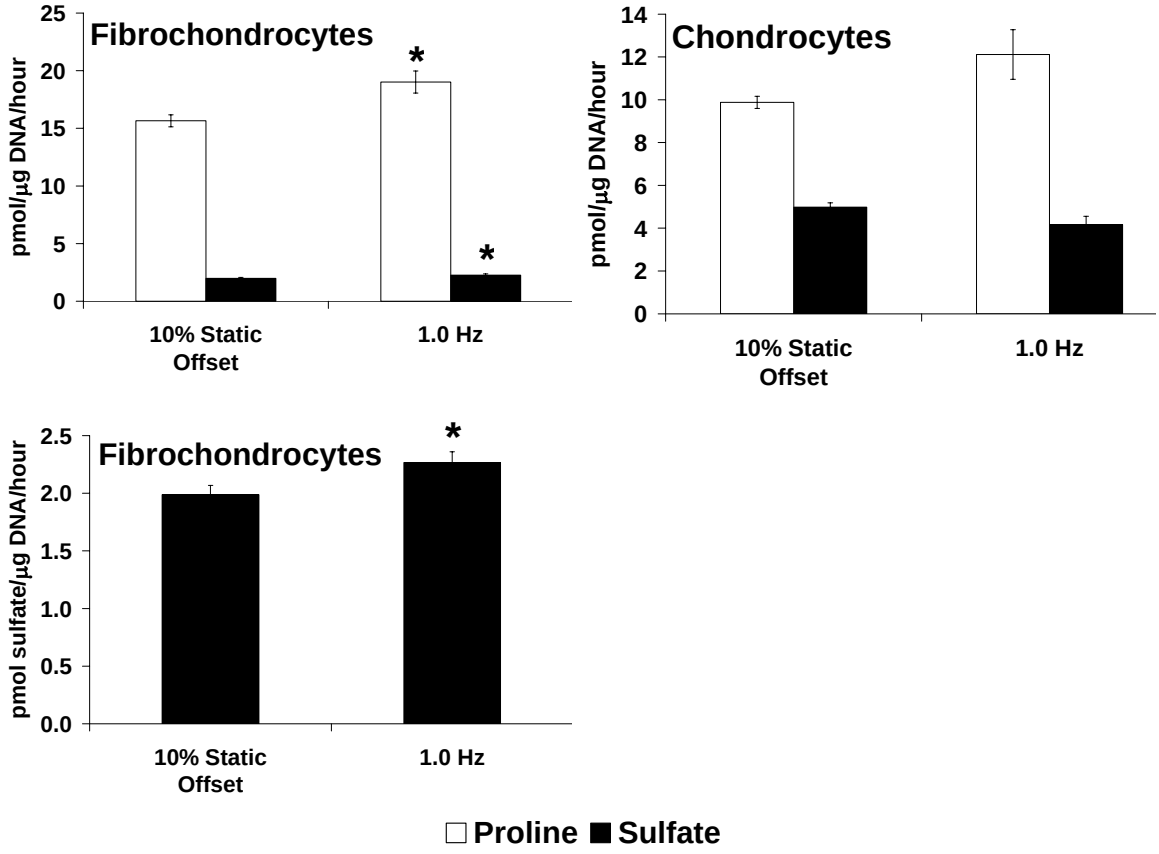

Figure 23: Effects of intermittent oscillatory compression (10% static offset ± 3% at 1.0 Hz) on agarose gels seeded with fibrochondrocytes or articular chondrocytes. Bottom graph shows fibrochondrocyte sulfate incorporation for scaling purposes. * indicates significant difference from 10% static offset ($p<0.038$). [n=8 per compression condition per cell type]

4.4 DISCUSSION

External biomechanical stimuli were shown to modulate the biosynthetic responses of meniscal fibrochondrocytes in native explant culture and agarose gel culture. These responses had similarities as well as several notable differences in comparison to the responses of articular chondrocytes subject to the same mechanical stimuli. Overall static compression of all tissue explants applied over a 21 hour period inhibited matrix production of both protein and proteoglycans. Comparing incorporation rates between the tissues, proline incorporation rates were on the same order of magnitude (with the chondrocytes presumably producing collagen type II and the fibrochondrocytes producing collagen type I). The relative inhibitions as percentages of the free swell controls were also comparable suggesting that the cells have similar mechanisms of sensing and responding to static compression. One proposed mechanism is cell-matrix interactions. Since the cells are intimately attached to and surrounded by matrix components, sustained deformations due to static compression could change cell shape. Chondrocyte deformation in tissue explants has been quantified using autoradiography and stereology[117,118]. With increases in static compression levels, cell and nucleus volumes and cell surface areas decreased, correlating with decreases in sulfate incorporation rates, showing a relationship between cell morphology and matrix synthesis. A second proposed mechanism by which the cells can sense static loading is through the local cellular environment. As the explants are compressed, fluid exudation and matrix compaction occurs. This leads to an increase in fixed charged density as the negatively charged glycosaminoglycan chains are packed closer together, in turn increasing the density of positive counterions and osmotic pressure. These intratissue

changes lead to a decrease in extracellular pH, a factor shown to affect matrix synthesis rates of chondrocytes[119]. A third mechanism is related to a transport-limitation of molecules (nutrients sulfate, waste products, etc.) through the compacted matrix. In order to assess if this was a major factor in the inhibition of matrix synthesis in the explants, further studies examining spatial differences in synthesis rates across the sample diameter would need to be performed. Kim *et al.* have shown that diffusive transport did not contribute to synthesis inhibition of their smaller (3 mm diameter) articular cartilage explants under increasing levels of static compression by showing no differences in inhibition for a specific compression level for disks of 2 or 3 mm in diameter[72].

Static compression of agarose gels seeded with either fibrochondrocytes or chondrocytes did not show inhibition of either protein or proteoglycan production for compression up to 50% of the thickness relative to free swell controls. In the gel studies, it is important to note that the control media condition was the basal media formulation containing 0.1% BSA versus the 10% FBS condition of the tissue explant studies. It was thought that using 0.1% BSA only as the basal media condition would allow for the isolation of the effects of compression alone. Compression of the gels was performed after a total of 10 days of culture (3 days of preculture included). As seen in the following chapter, the agarose gels seeded with either cell type at this time point in the BSA control conditions contains the lowest amount of sGAG as compared to other media conditions containing either TGF-β1, IGF-I or 10% FBS. The cells also are at a disadvantage as they are shown to have the lowest incorporation rates of both proline and sulfate in comparison to other, supplemented media conditions. Therefore, the serum-free medium condition may not be the best choice for looking at the effects of mechanical

stimuli, since the unsupplemented environment may not have the nutrients necessary to functionally regulate matrix synthesis in response to mechanical compression.

The current static compression results were similar to what was seen for chondrocytes seeded in 3% agarose and placed under graded levels at various time-points during matrix maturation in media supplemented with 10% FBS[73]. Buschmann *et al.* found that gels at early time points did not respond to static compression. Only after 13 days of culture was there a significant inhibition of matrix synthesis seen unnder approximately 36% compression. In contrast to this response to static loading, agarose gels as early as 2 days in culture were shown to respond to oscillatory compression with increases in matrix synthesis. In the current studies, oscillatory compression of the chondrocyte seeded agarose gels cultured in basal media did not stimulate matrix synthesis.

From the previous discussion of the response of the tissue explants, another contributor to the unresponsiveness of the fibrochondrocytes in agarose gels is the lack of appreciable amounts of matrix in the gels at the time of static loading. The lack of matrix could dictate the cellular behavior in two ways. First, any cell-matrix interactions are occurring between the cells and a non-functional matrix. As measured by Buschmann *et al.*, the mechanical properties of agarose gels seeded with chondrocytes approaches values of only 25% that of native cartilage after a month in culture[87]. Second, any changes in matrix synthesis due to local cellular environments (i.e., changes in fixed charged density leading to reduced pH, due to matrix compaction) will be decreased based upon the lower quantity of surrounding matrix. Lower concentrations of

glycosaminoglycans extracellularly would lead to smaller changes in fixed charged density under compression.

The application of low amplitude oscillatory compression has been shown to increase both proteoglycan and total protein synthesis by articular chondrocytes in explants[69,70,72] and agarose gel culture[73,74,77]. Sah *et al.* reported differences in synthesis rates due to a range of frequencies and oscillatory strain amplitudes[69]. A cutoff frequency of 0.001 Hz divided the frequency ranges into high and low realms. The low frequency region (0.0001-0.001 Hz) had physical characteristics of slow fluid exudation and minimal intratissue pressures, fluid velocities, and streaming potentials. Loading in this region only stimulated protein and proteoglycan production with high amplitude oscillatory strains. The high frequency region (0.01-1 Hz) had physical characteristics of increased internal hydrostatic pressure with a high pressure gradient across the radius of the tissue disk. In this region, significant increases in protein and proteoglycan production were seen for compression at all amplitudes greater than 1%. Another study within the same research group later showed that there was a localization of matrix synthesis in the same cartilage model. At frequencies equaling 0.1 Hz and greater, there was localized matrix synthesis stimulation in the outer 1 mm of the tissue explants. This localization was proposed to be related to increases in fluid flow and in changes in cell shape that are limited to the outer ring of the explants. As noted earlier, these cutoff frequencies change based on the culture environments studied, as they are dependent on matrix material properties and overall sample dimensions. A limitation of the current study is that the tissue samples were analyzed as wholes, neglecting the potential

differences in biosynthesis across the diameter. However, localization of biosynthesis due to physical phenomena will be discussed in Chapter 6.

In the current study, the application of what could be considered medium amplitude (3%) oscillatory compression at frequency of 1.0 Hz stimulated protein synthesis of both cartilage and meniscus tissue explants. The effect of this stimulus on proteoglycan synthesis varied between tissues. Agreeing with previous reports, there was stimulation of cartilage explants however; there was no effect on proteoglycan synthesis of meniscus tissue explants. One potential explanation for this finding is that the fibrochondrocytes respond at a lower level to oscillatory loading with changes in proteoglycan synthesis. However, adult porcine menisci have been shown to increase proteoglycan synthesis in response to 0.5 Hz illustrating age and/or species differences in response to mechanical compression[52].

There are several additional potential explanations for this discrepancy in behavior. First, it is possible that since fibrochondrocytes incorporate sulfate at such low rates as compared to the rates of incorporation for the chondrocytes, the data are approaching a lower limit of the ability to resolve differences in raw counts per minute. This is unlikely based upon the static loading data that showed low levels of overall sulfate incorporation rates. Despite these low levels, there were discernable differences between 25% compression and free swell controls.

The second proposal is that the fibrochondrocytes do not have the capacity to increase their proteoglycan synthesis over the low level of production that is seen in the free swell controls. To explore this, we used an alternative method of proteoglycan stimulation. IGF-I is a known stimulator of proteoglycan synthesis of chondrocytes and

has been identified in serum as one of the major factors that is responsible for the homeostatic turnover of proteoglycans in the cartilage matrix[7,10,79]. Exposure of meniscus tissue explants to concentrations of IGF-I up to 300 ng/mL stimulated sulfate incorporation rates by nearly 400% (Figure 24). This result showed that fibrochondrocytes do have the ability to increase proteoglycan production in response to an external stimulus, here a biochemical factor. This also led to the study of the effects of other growth factors on matrix biosynthesis as described in Chapter 5.

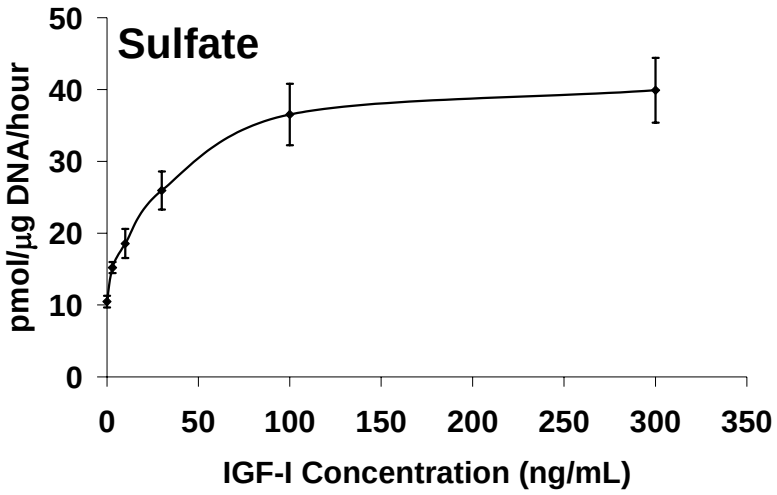

Figure 24: Dose-response of meniscus tissue explants for varying concentrations of IGF-I. [n=8 per concentration]

The last proposed idea to explain the lack of response in proteoglycan production of fibrochondrocytes to oscillatory compression was that the tissue was not undergoing the prescribed sinusoidal deformation. Since the sinusoidal load profile was prescribed based upon displacement control, and the system did not have the capability of recording

load in response to the prescribed displacement, a question concerning the actual loading profile arose. To determine the load response of the meniscus tissue under the prescribed displacement, samples were tested in the EnduraTEC ELectroForce 3200. A protocol in the Wintest software was set up to replicate the displacement controlled sinusoidal waveform. The program ramped down to the static offset position and held that position for 30 minutes, representing experimental setup time. Oscillatory compression with a 3% amplitude was then imposed with data acquired every at the end of 30 minute blocks of time throughout the 21 hour loading duration. Figure 25 depicts a representative graph showing the prescribed displacement as a sinusoidal compression and the non-sinusoidal recorded load. Any point above the dashed line (loads greater than 0 N) is a result of the impermeable platen "lifting-off" from the sample. For this sample loaded at 1.0 Hz, 10% ± 3%, the platen was not in contact with the sample for approximately 42% of the loading cycle. In contrast, "lift-off" was not detected during the loading of cartilage explants. Multiple additional tests were performed on meniscus tissue explants with frequencies in the range of 0.1 – 1.0 Hz and static offsets of values up to 25% of the thickness. For all tests, the relaxation of the tissue matrix during the loading protocol induced "lift-off" for all combinations studied. Therefore, the difference in the input compressive protocol and the actual loading protocol that the meniscus tissue sees could be responsible for the insensitivity in proteoglycan production, as the tissue is only actually loaded during 40% of the time.

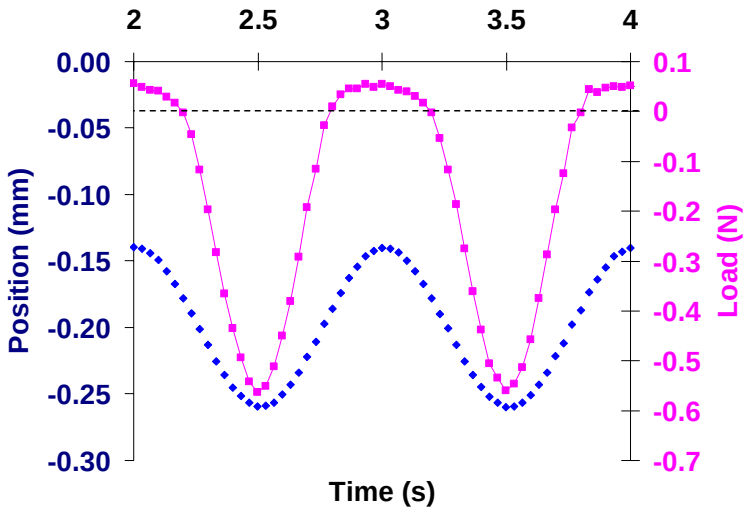

Figure 25: Representative data acquired from the ELectroForce 3200. This data is from the testing of a meniscus tissue explant of dimensions 4 mm diameter x 2 mm thick subject to sinusoidal oscillatory compression (10% ± 3%). For the prescribed sinusoidal displacement, there is a non-sinusoidal load response. The points above the dashed line having positive load values represent the impermeable platen "lifting-off" from the tissue sample.

After determining the actual waveform of the oscillatory compression on the meniscus tissue explants, the question of why the protein synthesis was preferentially stimulated by this waveform arose. Studies using oscillatory protocols with slight variations in static offsets and/or compressive amplitudes have detected "lift-off" during their loading cycles[77,120]. However, the use of these protocols was justified by identifying that the dynamic component to loading was present throughout the loading duration. These studies showed increases in chondrocyte matrix synthesis with oscillatory loading. This suggests that the non-continuous loading protocols, similar to the one used in the current studies, still have the capacity to stimulate cells to increase matrix synthesis. The differences in the fibrochondrocyte protein and proteoglycan synthesis in response to

non-continuous oscillatory loading are suggestive of different thresholds of stimulation needed for proteoglycan synthesis.

Different combinations of compressive static offset (up to 25%), amplitude (1.5% to 3%), and frequency (0.1, 0.5, 1.0 Hz) were examined to find a fully continuous loading protocol for the meniscus explants. No combination of these parameters yielded a loading protocol without "lift-off." In many cases, "lift-off" occurred within 10 minutes of the commencement of the oscillatory loading protocol. Therefore, the studies shifted towards using fibrochondrocytes in 3-D gel culture to explore the effects of biomechanical stimuli on matrix biosynthesis. In order to evade the "lift-off" issue that was seen with the tissue explants, several formulations of agarose gels were tested on the ELectroForce 3200 using the same protocol. Agarose gels of 3% (w/v) and dimensions of 6 mm diameter x 3 mm were able to maintain a sinusoidal load profile throughout a 21 hour loading period. Therefore these compositional and geometrical parameters were used in the final studies looking at the effects of oscillatory compression on the cells in agarose gels.

The intermittent oscillatory compression protocol was taken from the work of Chowdhury *et al.*[77]. Their work looked at the effect on chondrocyte biosynthesis in agarose gels due to a variety of loading protocols including both continuous and intermittent loading. One finding that is relevant to the current work was the identification of the maximum number of cycles within a specified time period that will induce maximal stimulation of proteoglycan synthesis as compared to unstrained controls. They found that spreading out a 24 hours of loading over a 48 hour time period had the greatest increases of proteoglycan synthesis levels over any other regime studied.

Therefore, this intermittent protocol was chosen as the loading protocol for the current studies.

The results for the intermittent oscillatory loading of the agarose gels seeded with fibrochondrocytes or chondrocytes showed differences in the effects on biosynthesis of the two cell types studied. The lack of stimulation of the chondrocytes may be due to differences in fluid flow from what has been previously shown by Buschmann *et al*. It is known that the fluid velocity is proportional to the product of the aggregate modulus (H_A) and hydraulic permeability (k) of the explant or gel of given dimensions[78]. Although these material properties were not measured for our samples, pooling data from the literature, the differences in gel dimensions would create fluid velocities that differed from what was seen by Buschmann *et al.* that could be responsible for the lack of response in matrix synthesis. This suggests that the chondrocytes and fibrochondrocytes of these studies have distinct responses to the same fluid regimes, as the fluid flow in both gels were comparable based on similar dimensions and their early stages of matrix deposition with likely similar material properties.

Since these gels were cultured in the absence of any stimulatory factors, the contribution of increased transport of soluble factors from the surrounding medium environment is not likely to stimulate biosynthesis of the cells in gel culture. It is likely then that the weak cell-matrix interactions in comparison to the interactions in native tissue are the main contributors to the non-responsiveness of the chondrocytes. In the 10 day old agarose gel, the surrounding matrix produced by the chondrocytes is mainly pericellular. With the deformation of the matrix, it may be the scaffold itself that is going

through the greatest strain, minimally affecting the cells and their pericellular surroundings.

Unlike the static compression studies, there was a significant effect of oscillatory compression on stimulating biosynthesis of fibrochondrocytes. We cannot make a direct connection between the effects of loading in gels versus loading in tissue explants. Since the application of oscillatory loading on the tissue featured "lift-off" of the impermeable platen, the mechanical environment was not limited to radial transport only and the matrix experienced variable strains throughout the loading over 21 hours due to relaxation of the tissue matrix. The stimulation of biosynthesis in the fibrochondrocyte seeded gels may be due to the cells actually sensing the appropriate fluid flow obtained only in the agarose gel system under continuous application of oscillatory compression. The response of fibrochondrocytes in response to fluid shear is unknown. However, it has been shown to induce chondrocyte production of matrix molecules and stimulatory factors, suggestive of the importance of fluid shear in homeostatic maintenance of cartilage[121].

In conclusion, mechanical compression has been shown to modulate matrix biosynthesis of fibrochondrocytes and chondrocytes in tissue explants. For both tissues, with increasing levels of static compression there were relative increases in inhibition of matrix synthesis rates. Relative baseline sulfate incorporation rates were over an order of magnitude lower in the native fibrochondrocytes as compared to the native chondrocytes. When placed in agarose gels, matrix synthesis was not inhibited in response to static compression, suggesting a role of cell-matrix interactions in sensing mechanical compression. In response to a displacement controlled oscillatory loading protocol, both

cell types in explant culture responded with stimulation of proline incorporation rates. However, only chondrocytes had stimulated sulfate incorporation rates in response to oscillatory compression. When seeded into agarose gels, fibrochondrocytes showed increased matrix synthesis, whereas chondrocytes were insensitive to oscillatory compression. These findings show differences in cell behaviors that appear to be a function of matrix environment.

CHAPTER 5

DIFFERENTIAL EFFECTS OF ANABOLIC CYTOKINES ON THE BIOSYNTHESIS OF MENISCAL FIBROCHONDROCYTES IN EXPLANTS AND AGAROSE GEL CULTURE

5.1 INTRODUCTION

In addition to biomechanical cues, as explored in Chapter 4, the development, maintenance, and repair of the meniscus also relies upon biochemical cues. These cues may be autocrine, paracrine, or endocrine in nature[122-124]. Additionally, these chemicals, broadly termed as cytokines, can have anabolic or catabolic effects on the target cells in tissues. In some cases, a single factor has the potential to be either anabolic or catabolic depending upon the environment that it is in or its local concentration. These studies examined the individual effects of several cytokines on the biosynthesis of fibrochondrocytes in native tissue explants and agarose gel culture.

The cytokines chosen for these studies were under the sub-category of growth factors (typically anabolic in action) and are show in Table 3 with the recombinant human forms of each factor used. The cytokine concentration ranges chosen (Chapter 5.2, Table 4) were based on published studies on articular cartilage explants and chondrocytes in alginate beads and monolayer culture[7-13], as discussed in Chapter 2. These factors in concert with catabolic cytokines direct normal homeostatic turnover of orthopaedic tissues. Additionally, these factors have been detected in elevated quantities in diseased states such as osteoarthritis and rheumatoid arthritis, and are involved in triggering matrix degradation events[123-125].

Supplementation of cytokines to a media formulation containing FBS is commonly used; however in this formulation a known cytokine concentration is being added to the uncharacterized mixture of cytokines present in the FBS. Since the actions of a single growth factor can be highly influenced by the presence of other factors (anabolic or catabolic) in the culture environment, each factor was studied individually in a serum-free culture environment containing 0.1% bovine serum albumin (BSA), a protein that acts as a carrier aiding in the delivery of the growth factors to the cells. Comparison of these results with other studies will depend upon the culture conditions used.

We hypothesized that the addition of these individual growth factors would increase matrix synthesis of fibrochondrocytes in native tissue culture explants as well as accelerate matrix accumulation in agarose gels seeded with fibrochondrocytes. First, the biosynthetic response of meniscal explants to a range of concentrations of each factor was studied. A saturation of response to each factor was expected at the upper limits of the chosen concentration ranges. Next, a single concentration of each factor was chosen, and the effects of long-term (up to 14 days) culture on tissue explants were studied. We hypothesized that there would be a sustained effect of continued exposure to these factors, with stimulation in biosynthesis that reached a constant level after a certain time within the culture duration. Finally, the individual effects of TGF-β1 and IGF-I on matrix accumulation, synthesis rates, and gene expression by fibrochondrocytes and chondrocytes in agarose were compared over a 14 day culture period. These two factors have been identified as mediators of directing the differentiation and matrix production of chondrocytes[79,81,126,127]. We hypothesized that addition of these growth factors would

accelerate matrix deposition by both cell types in gel culture. The findings from these studies will aid in understanding the effects of individual growth factors on fibrochondrocytes in two culture systems, hinting at the role of each factor in tissue maintenance and matrix generation. Additionally, the agarose gel studies will allow us to directly compare the effects of the same biochemical stimuli on cells from cartilage and fibrocartilage.

Table 3: List of growth factors used in these studies. All growth factors were obtained in their recombinant human forms as lyophilized powders.

Growth Factor	Abbreviation	Size (kDa)
Fibroblast Growth Factor-basic	bFGF	17.2
Insulin-like Growth Factor-I	IGF-I	7.6
Platelet-Derived Growth Factor-AB	PDGF-AB	25.5
Transforming Growth Factor-beta 1	TGF-β1	25.0

5.2 MATERIALS AND METHODS

5.2.1 Tissue and Cell Harvest Procedure

Meniscus tissue explants with dimensions of 4 mm in diameter by 1 mm in thickness were used for these studies. The articular chondrocytes and fibrochondrocytes were cast into 3% agarose gels at 5 x 10^6 cells/mL. Tissue and cell isolation procedures are detailed in the previous chapters. Explants and agarose gels were precultured in basal/serum-free medium (DMEM plus 0.1% BSA, 0.1 mM NEAA, 1.0 mM HEPES, 50

μg/mL gentamicin, 0.25 μg/mL fungizone, 0.4 mM L-proline and 50 μg/mL ascorbate) for three days following excision or casting.

5.2.2 Dose-Response Studies

Tissue explants only

These studies examined the response of meniscus explants to simulation with a range of concentrations of each cytokine (Table 4). Four concentrations of each cytokine spanning two orders of magnitude were chosen based on previous studies in the literature involving cartilage tissue or isolated chondrocytes [7-13]. Four separate "sub-studies" were conducted, each of which utilized tissue from a different animal and included a control explant group cultured in basal medium alone (BSA controls). Study 1a included treatments with bFGF at 1-300 ng/mL (0.0581-17.4 nM), IGF-I at 30-1000 ng/mL (3.95-132 nM), or TGF-β1 at 0.05-5 ng/mL (0.002-0.2 nM). Study 1b included treatments with IGF-I at 30-1000 ng/mL or PDGF-AB at 3-300 ng/mL (0.118-11.8 nM). Study 1c included treatments with all four growth factors at the previously stated concentration ranges. Finally, study 1d was performed in order to explore a higher range of concentrations of TGF-β1 at 1-100 ng/mL (0.04-4.0 nM) based on the results of the first dose-response studies.

For all studies, following the initial preculture in basal medium, the tissue explants (4 mm diameter x 1 mm thick, n=6 per condition per study, total n=12-18 per condition) were cultured for 4 days in 1.0 mL each of appropriately supplemented medium. The media were supplemented with 20 μCi/mL of L-5-^{3}H-proline and 10 μCi/mL of ^{35}S-sodium sulfate for the final 21 hours of the 4 day culture period. At the end of the radiolabel period, samples were washed to rinse out unincorporated

radiolabeled precursors. The digestion and scintillation counting protocols as described in the previous chapter were followed.

Table 4: Cytokine concentrations used for the meniscus tissue explant dose-response and time-course studies.

Growth Factor	Dose-Response (ng/mL)				Time-Course (ng/mL)
bFGF	1	10	100	300	100
IGF-I	30	100	300	1000	200
PDGF-AB	3	30	100	300	100
TGF-β1*	0.05	0.5	1	5	5

** A second dose-response study was performed for TGF-β only using a higher range of concentrations (1, 5, 10, 50, and 100 ng/mL).*

5.2.3 Time-Course Studies

Tissue explants

These studies compared the duration of stimulation of meniscus tissue explants (4 mm diameter by 1 mm thick) by a single concentration (Table 4) of each cytokine for up to two weeks. This single concentration was chosen based upon the concentration that had the greatest stimulatory effect on matrix synthesis in the dose-response studies. After preculture, tissue explants from a single animal (n=5 per condition per time point) were cultured for 2, 4, 7, or 14 days in basal medium plus a single cytokine at the chosen concentration. Control explants were cultured in basal/serum-free medium alone (BSA controls). Radiolabeled precursors were added at the aforementioned concentrations for the final 21 hours of each time point.

Agarose gels

These studies compared the responses of fibrochondrocytes and articular chondrocytes seeded in agarose gels to either 5 ng/mL of TGF-β1 or 200 ng/mL of IGF-I for up to two weeks. The concentrations of this sub-set of cytokines matched the values used in the tissue explant time-course studies. After preculture in basal/serum-free medium containing 0.1% BSA, gels were cultured for 14 days in one of three media conditions: basal medium + 5 ng/mL TGF-β1, basal medium + 200 ng/mL IGF-I, or basal medium alone. Gels were also cultured in a fourth media condition containing 10% FBS (DMEM plus 10% FBSA, 0.1 mM NEAA, 1.0 mM HEPES, 50 μg/mL gentamicin, 0.25 μg/mL fungizone, 0.4 mM L-proline and 50 μg/mL ascorbate). Media were changed ever other day, and the samples were taken down at days 1, 2, 4, 7, and 14.

Two separate "sub-studies" were conducted, each of which utilized tissue from a different animal. The first study (n=8 per media condition per day for fibrochondrocytes; n=4 per condition per day for chondrocytes) was a non-radiolabel study, where samples were used for analysis of sulfated glycosaminoglycan and collagen contents. In the second study (n=8 per media condition per day for both cell types), radiolabeled precursors at concentrations of 10 μCi/mL of L-5-^{3}H-proline and 5 μCi/mL of ^{35}S-sodium sulfate were added for the final 21 hours of each time point to measure total protein and proteoglycan synthesis, respectively.

Real-Time Quantitative RT-PCR

In a separate study utilizing cells from a separate donor, agarose gels seeded with chondrocytes and fibrochondrocytes were prepared and cultured for two weeks in the same manner as previously described. At time-points of 1, 7, and 14 days, samples were

harvested for RNA isolation (n=3-4 per media condition per day per cell type). In pilot studies, it was determined that a minimum cell number was necessary to obtain an accurately detectable amount of RNA. Therefore, each sample was a collection of 3 gels from the same media condition. These pooled samples were immediately placed in 450 μL of RLT lysis buffer and melted at 60°C for 10 minutes. Samples were then place in the -80°C freezer for subsequent RNA isolation (see Chapter 3 for details). Gene expression for collagen types I and II and aggrecan were examined.

5.3 RESULTS

5.3.1 Dose-Response Studies

Tissue explants

In general, the tissue explants responded to increasing levels of each growth factor with a dose-dependent stimulation of matrix production. Figure 26 illustrates the dose-responses of both proline and sulfate incorporation rates for all growth factors normalized by the average of the BSA controls.

For IGF-I and PDGF-AB, the dose-responses appeared to plateau within the chosen concentration ranges for both the proline and sulfate incorporation rates. IGF-I obtained maximal levels of stimulation by 300 ng/mL with sulfate and proline incorporation rates reaching 291% and 188% of BSA controls, respectively. The sulfate incorporation at 300 ng/mL IGF-I was significantly higher than incorporation at 0 ($p<0.0001$), 30 ($p<0.0001$), and 100 ng/mL ($p=0.0040$). The proline incorporation at 100

ng/mL IGF-I was significantly higher than at 0 ng/mL ($p=0.012$), and at 300 ng/mL IGF-I was significantly higher than at 0 ($p<0.0001$) and 30 ng/mL ($p=0.0008$). PDGF-AB obtained maximal levels of stimulation at 100 ng/mL with sulfate and proline incorporation rates reaching 468% and 148% of BSA controls, respectively. These maximal levels of stimulation were both significantly higher than incorporation rates at any of the lower concentrations (all $p<0.0002$).

In contrast to IGF-I and PDGF-AB, the initial concentration range chosen for TGF-β1 (0.05 – 5 ng/mL) did not appear to contain saturated incorporation of either sulfate or proline (Figure 26 bottom left, open markers). The sulfate incorporation increased monotonically with TGF-β1 concentration and still appeared to be increasing at 5 ng/mL, the highest level in the initial dose-response study. At 5 ng/mL, the sulfate incorporation rate was 640% of the BSA control value and was significantly greater than incorporation at any lower concentration (all $p<0.0001$). In contrast, the proline incorporation rate at 5 ng/mL was 161% of the BSA control value and was only significantly higher than at 0 ($p<0.0001$) and 0.05 ng/mL ($p=0.0003$). When the concentration range was expanded to include concentrations of up to 100 ng/mL, maximal levels of sulfate and proline incorporation were seen at 100 ng/mL, reaching 1034% and 319% of BSA control values, respectively (Figure 26 bottom left, solid markers). The sulfate incorporation at 100 ng/mL TGF-β1 was significantly higher than at 0 ($p<0.0001$), 1 ($p<0.0001$), 5 ($p=0.0070$), and 10 ng/mL ($p=0.0027$). The proline incorporation at 100 ng/mL TGF-β1 was only significantly higher than incorporation at 0 ($p<0.0001$) and 1 ng/mL ($p=0.0003$). However, the response to TGF-β1 still did not appear to be truly saturated by 100 ng/mL.

Finally, bFGF showed modest stimulation of sulfate incorporation and no stimulation of proline incorporation over the chosen concentration range. The sulfate incorporation at 100 ng/mL bFGF was significantly higher than at 0 ng/mL ($p=0.0028$), and at 300 ng/mL was significantly higher than at 0 ($p<0.0001$), 1 ($p=0.0072$), and 10 ng/mL ($p=0.017$). The greatest sulfate incorporation occurred at 300 ng/mL, reaching 169% of the BSA control value with no clear plateau in stimulation. At these concentrations, bFGF did not significantly affect proline incorporation ($p=0.19$).

Overall, the growth factors chosen were more potent stimulators of sulfate incorporation than of proline incorporation. There were different levels of stimulation for the four factors chosen. TGF-β1 was the most potent stimulator of sulfate incorporation with an order of magnitude increase in incorporation rate at the highest concentration. bFGF was the least potent stimulator of both sulfate and proline incorporation over the ranges studied.

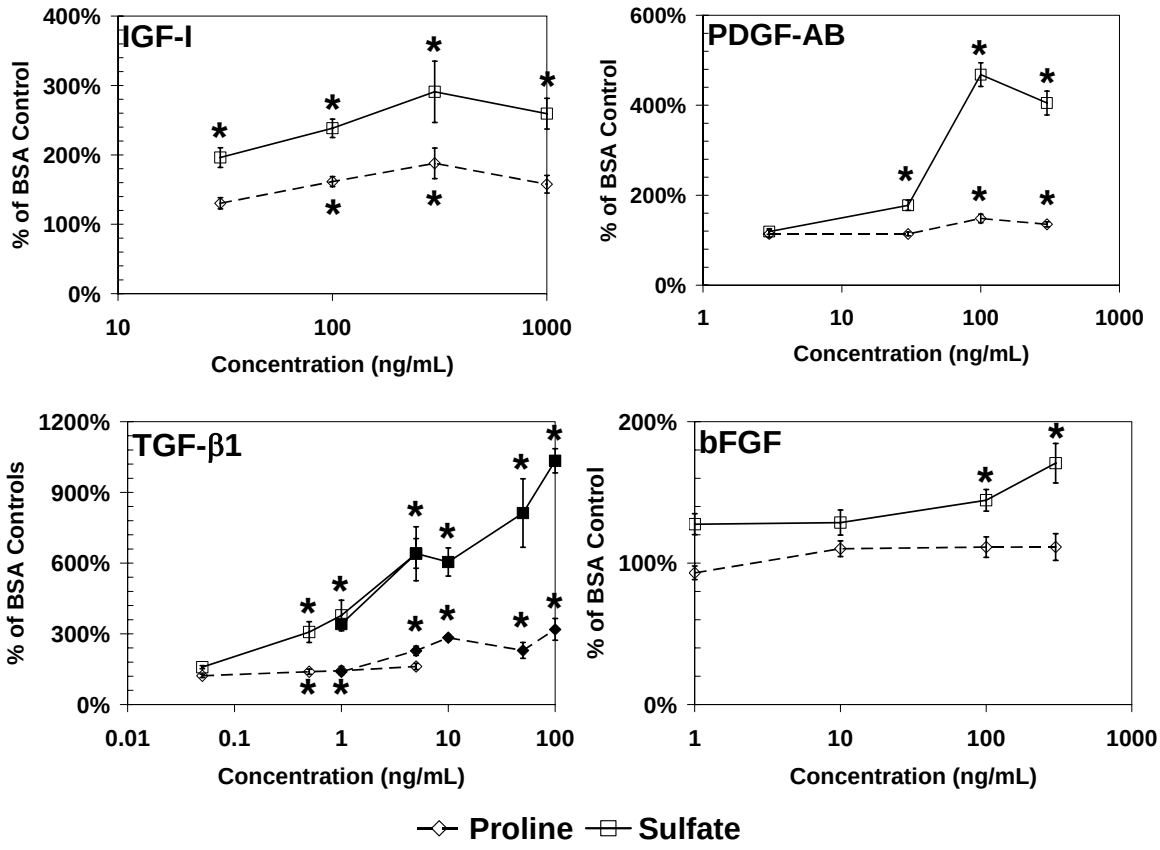

Figure 26: Dose-response results for proline and sulfate incorporation rates of meniscus tissue explants for a range of concentrations of IGF-I, PDGF-AB, TGF-β1, and bFGF. For presentation purposes, data are normalized to basal/serum-free (BSA) control values. For TGF-β1 (bottom left) solid markers represent data from the second dose-response study. * indicates significant difference from BSA controls ($p<0.05$). [n=12-18 per cytokine concentration]

5.3.2 Time-Course Studies

Tissue explants

Based on the results of the initial dose-response studies, single concentrations of each growth factor (Table 4) were chosen to examine the effects of these factors over a 14 day period. None of the growth factor groups exhibited proline incorporation significantly different from the BSA controls ($p=0.078$ to $p=0.99$, Figure 27), although the proline incorporation for the PDGF-AB group was significantly less than that of the TGF-β1 ($p=0.0063$) and IGF-I groups ($p=0.0055$). The proline incorporation at day 7 was also significantly lower than at all other days (all $p<0.001$). In contrast, a significant and sustained stimulation of sulfate incorporation over BSA controls was seen for TGF-β1, IGF-I, and PDGF-AB (all $p<0.0001$), but not for bFGF ($p=0.51$). A significant dip in sulfate incorporation was seen at day 7 ($p=0.0031$ vs. day 4 and $p=0.024$ vs. day 14) for the TGF-β1 and PDGF-AB groups, but this was not sustained (incorporation at day 14 was not significantly different from that on days 2 or 4, $p>0.90$).

As only a fraction of the newly synthesized matrix molecules was incorporated into the tissue explants, we also quantified the total sGAG released to the media over the first 12 days (Figure 28). The cumulative sGAG release data for the TGF-β1 and PDGF-AB groups were generally consistent with the sulfate incorporation data, with 12 day cumulative sGAG releases of 221% and 133% of BSA levels, respectively. TGF-β1 supplementation induced the greatest 12 day cumulative sGAG release ($p<0.012$ vs. all other groups). Interestingly, however, the cumulative sGAG released for the IGF-I group was lower than that of the BSA group (73.2%), despite the fact that the IGF-I group had higher sulfate incorporation rates than the BSA controls. This suggests that the fraction

of newly synthesized sGAG incorporated into the extracellular matrix varied considerably among treatment groups, and that the growth factors may differentially influence assembly and processing of proteoglycans as well as the overall synthesis rates.

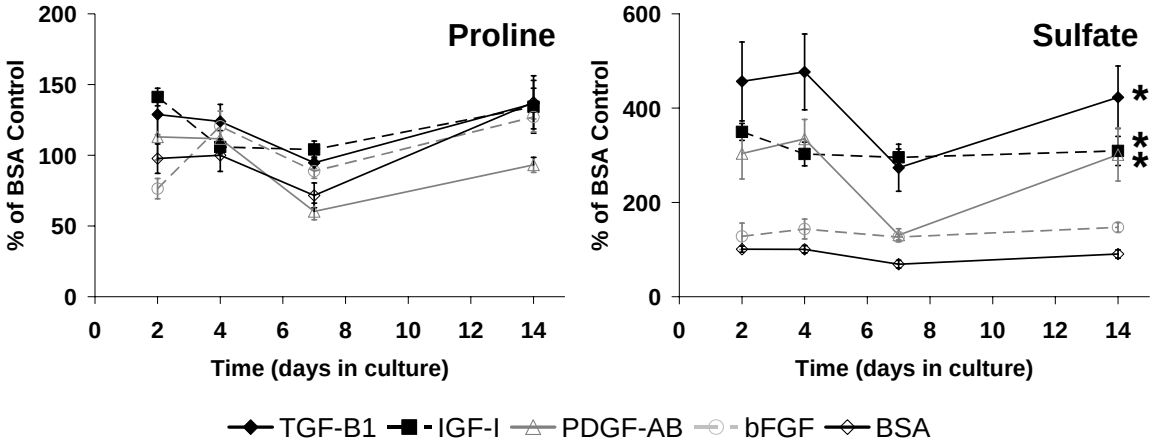

Figure 27: Time-course results for proline and sulfate incorporation rates of meniscus tissue explants over the two week culture period. For presentation purposes, data are normalized to baseline BSA control values at day 0 (following preculture but before adding growth factors). * indicates significant difference from BSA control ($p<0.0001$). [n=5 per cytokine concentration per time-point]

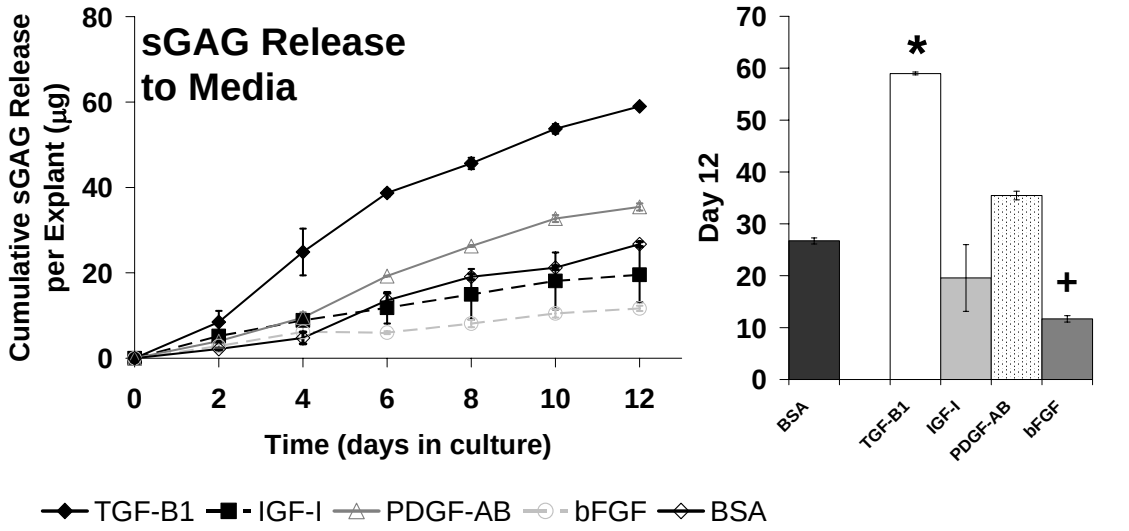

Figure 28: Cumulative release of sulfated glycosaminoglycans (sGAG) from meniscus tissue explants into the media over the two week culture period. The data are presented on a per explant basis. * indicates significant difference from all other media treatments ($p<0.012$). + indicates significant difference from PDGF-AB ($p<0.012$). [n=5 per media condition per time-point]

Agarose gels

General Biochemical Analysis

The DNA content of the gels seeded with fibrochondrocytes was not significantly affected by the different media conditions over the two week culture period ($p=0.055$, Figure 29). Day 1 gels had significantly higher levels of DNA than days 2, 4, and 7 gels ($p<0.036$), however after the first day of culture the DNA content was fairly constant through the rest of the culture period. The agarose gels seeded with chondrocytes had significant changes in DNA content due to both media condition ($p=0.011$) and culture time ($p<0.001$). FBS caused chondrocyte proliferation within the agarose gels, resulting in higher DNA content compared to BSA or TGF-β1 ($p=0.014$ and $p=0.030$, respectively). Overall, the DNA content was highest at day 14 compared to all other days of culture ($p<0.016$). This effect was primarily due to the greatest accumulation of DNA in the presence of FBS ($p<0.001$). In a model excluding the FBS group, there were no significant differences of DNA content among days or media conditions ($p>0.091$). Although the cytokines used in these studies have been implicated in cellular proliferation, for the agarose gel culture system presented, there was no significant effect on proliferation over 14 days.

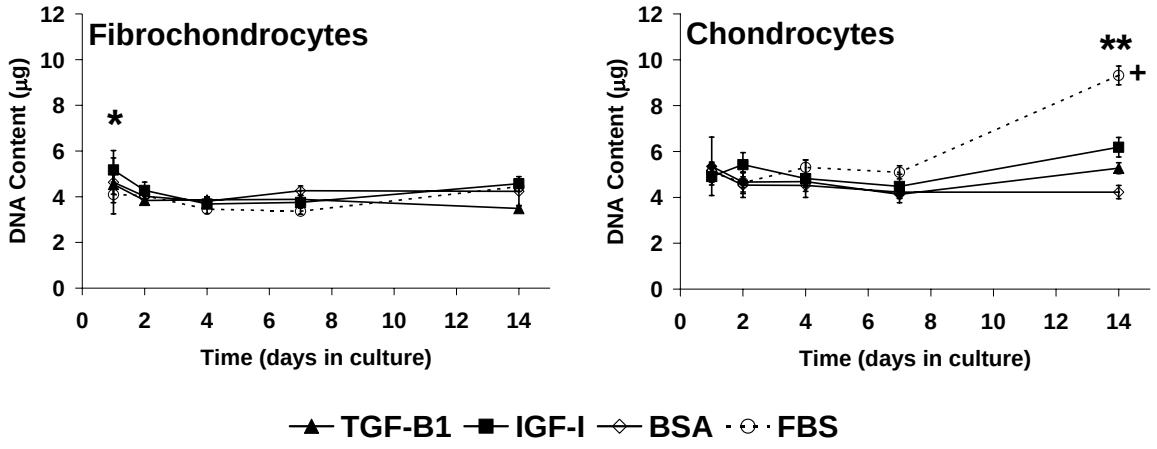

Figure 29: DNA content of agarose gels seeded with fibrochondrocytes or chondrocytes. * indicates significant difference from DNA contents at days 2, 4, and 7 ($p<0.036$). ** indicates significant difference from DNA content at all other time-points ($p<0.016$). + indicates significant difference in DNA content from TGF-β1 or BSA media conditions ($p<0.030$). [n=16 per media condition per time-point for fibrochondrocytes and n=12 per media condition per time-point for chondrocytes]

The accumulation of sGAG over the 14 day culture period was also quantified. The data were normalized to the DNA content within each gel (Figure 30). The fibrochondrocytes showed a significant and sustained stimulation of sGAG accumulation for TGF-β1 and FBS over both the control condition of BSA only and supplementation with IGF-I ($p<0.001$). TGF-β1 stimulated the greatest sGAG accumulation over all of the other media conditions ($p<0.001$). Overall at each time-point studied after day 2, the levels of sGAG accumulation were significantly greater than at the previous time-point ($p<0.0002$). The chondrocytes had a very different sGAG accumulation profile in response to the same media conditions. All media conditions had increased levels of sGAG accumulation on a per cell basis over the BSA control condition ($p<0.001$). The FBS group had the most significant amounts of sGAG production over either cytokine

group ($p<0.001$). Unlike the fibrochondrocytes, the increases in sGAG accumulation by day 14 appeared to plateau for all media treatments with sGAG contents that were not significantly different than what was measured at the previous time-point ($p=0.43$). It should be noted that the sGAG contents of chondrocyte gels were an order of magnitude higher than for fibrochondrocyte gels, with chondrocyte sGAG accumulation in the BSA gels comparable to that in the highly stimulated fibrochondrocyte gels.

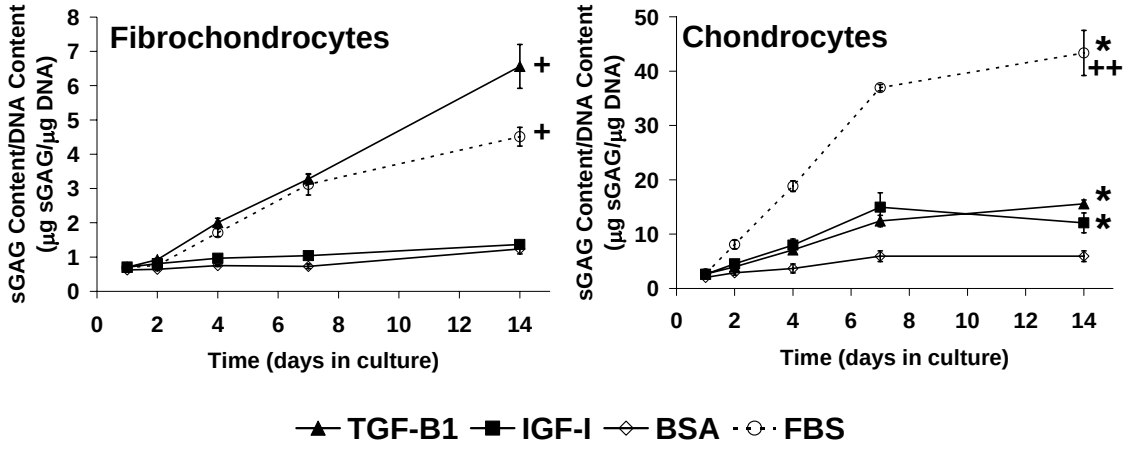

Figure 30: sGAG accumulation on a per cell basis of agarose gels seeded with fibrochondrocytes or articular chondrocytes. Note the difference in scales needed to display accumulation for the different cell types. + indicates significant difference in sGAG content from IGF-I and BSA media conditions ($p<0.001$). * indicates significant difference in sGAG content from BSA media condition ($p<0.001$). ++ indicates significant difference in sGAG content from TGF-β1 and IGF-I media conditions ($p<0.001$). [n=16 per media condition per time-point for fibrochondrocytes and n=12 per media condition per time-point for chondrocytes]

Media Analysis

Appreciable levels of matrix components were released to the media over the two week culture. For fibrochondrocyte gels, cumulative sGAG release for the FBS and TGF-β1 groups were significantly higher than for the BSA control gels ($p<0.001$, Figure 31). There was significant no difference in sGAG released into the media between the IGF-I and BSA groups ($p=0.43$). FBS caused the most significant release of sGAG into the media, releasing 175% of BSA control values ($p<0.0001$). Although treatment with TGF-β1 significantly increased sGAG accumulation within the gel, it appears that FBS caused greater overall sGAG production when both accumulation in the gel and expulsion into the media are accounted for. Similar to fibrochondrocytes, chondrocytes released the most sGAG into the surrounding media with FBS treatment, releasing 228% of BSA control values ($p<0.0001$). Each supplemented media group had a cumulative sGAG release that was significantly greater than the release of the BSA control gels ($p<0.0031$). Release profiles for all treatments were significantly different from each other ($p<0.0031$).

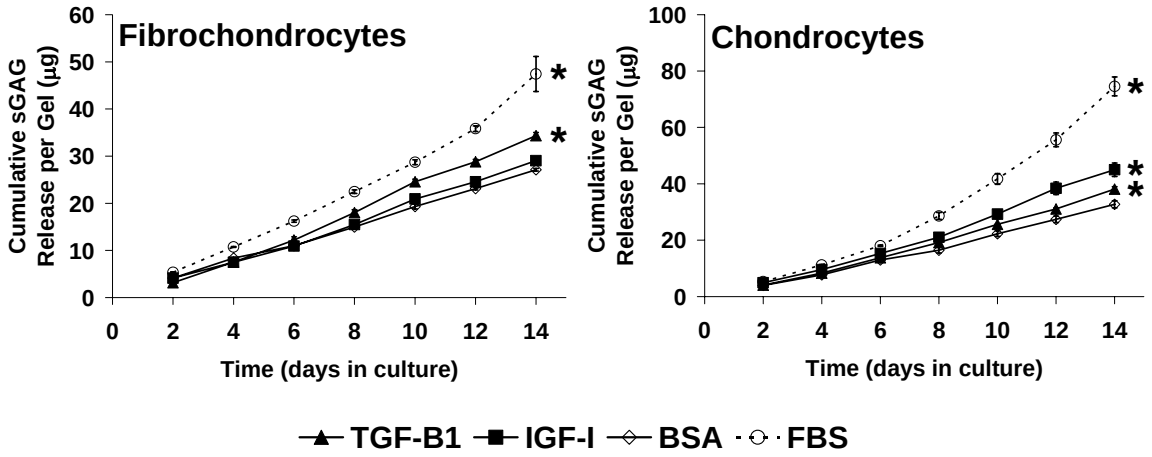

Figure 31: Cumulative release of sulfated glycosaminoglycans (sGAG) into the media by fibrochondrocyte and chondrocyte seeded agarose gels over the two week culture period. * indicates significant difference from sGAG release of BSA controls. The data are presented on a per gel basis. [n=4 per media condition per time-point]

Collagen release profiles as measured by the amount of hydroxyproline in the spent media were similar to the sGAG release profiles (Figure 32). For fibrochondrocyte gels, increased cumulative amounts of collagen were expelled into the media for the FBS and TGF-β1 groups as compared to the BSA control group ($p<0.0092$). There was no significant difference in the cumulative collagen amounts released to the medium between the IGF-I and BSA groups ($p=0.97$). FBS caused the most significant release of collagen into the media, releasing 186% of BSA control values ($p<0.0001$). On a daily basis for all time-points studied, the FBS groups released the greatest amount of collagen as compared to all other media conditions ($p<0.0001$). Similarly for chondrocytes, the FBS caused the greatest release of collagen as compared to all other media conditions ($p<0.001$). However unlike the fibrochondrocytes, there was a significantly greater

cumulative release of collagen seen in the BSA control groups over either of the cytokine groups ($p<0.0009$).

Currently the accumulation of collagen within the agarose gels has not been quantified. The collagen in the samples was difficult to extract reliably, resulting in large variations in the data. Mauck *et al.* have shown very low collagen contents for their chondrocytes seeded at twice the current cell density in 2% agarose at culture times through day 21, followed by a sharp increase in accumulation through day 35[16]. As our culture conditions contain even fewer cells, it is likely that the concentrations of collagen in this study were at the lower limits in detection of the assay.

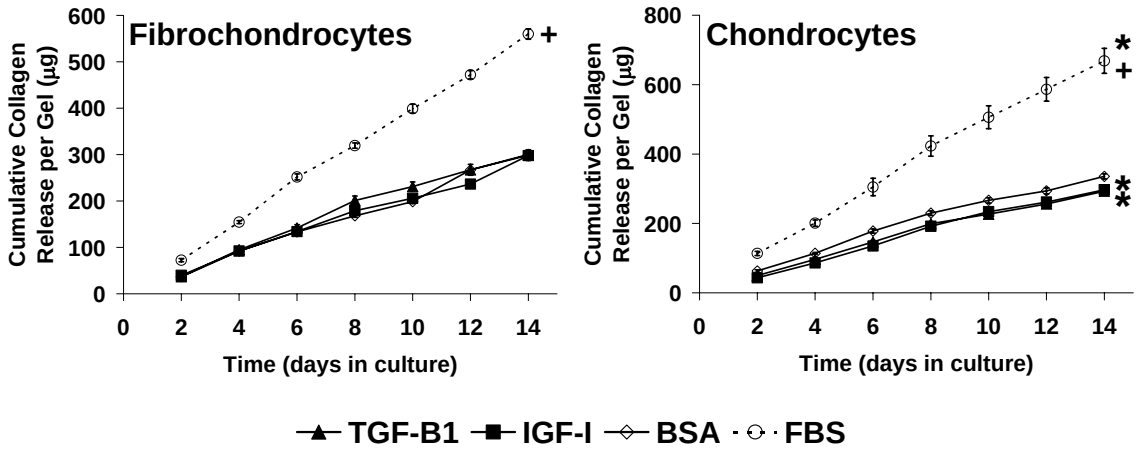

Figure 32: Cumulative release of collagen into the media by fibrochondrocyte and chondrocyte seeded agarose gels over the two week culture period. * indicates significant difference from BSA controls ($p<0.0092$). + indicates significant difference from all other media groups ($p<0.0001$). The data are presented on a per gel basis. [n=4 per media condition per time-point]

Radiolabel Incorporation

In addition to determining the general biochemical effects of the cytokines on fibrochondrocytes and chondrocytes in agarose gel culture, the effects on matrix synthesis rates were also determined. The biosynthesis data for fibrochondrocytes in agarose gels were found to be non-normal due to increased variability of the data with both time and media condition. To confer normality to the data set, a Box-Cox Transformation was performed on both proline and sulfate incorporation rates on a per cell basis using the optimum lambda coefficient determined by Minitab. The transformed data were put into a General Linear Model using a two-factor analysis with interaction term (media, day, and interactions between media and day). For incorporation of both proline and sulfate, there was a significant interaction that influenced the model that varied with both day and media condition ($p<0.001$).

All media conditions studied showed increased and sustained stimulation of fibrochondrocyte proline incorporation rates over BSA control values throughout the two week culture period ($p<0.0001$, Figure 33). This is in contrast to the proline incorporation rates of fibrochondrocytes in native tissue showing no significant differences dependent upon media condition or time in culture (Figure 27). TGF-β1 stimulated greater proline incorporation rates over those of any other media condition ($p<0.0046$). Additionally, all media groups stimulated significantly different rates of proline incorporation compared to each other ($p<0.0046$). There was a plateau effect seen when collectively looking at all factors; proline incorporation rates increased through day 4 ($p<0.028$), however after that time-point, there were no significant increases in incorporation rates between time-points studied ($p>0.53$).

Similarly all media conditions studied showed increased and sustained stimulation of fibrochondrocyte sulfate incorporation rates over BSA control values throughout the two week culture period ($p<0.0019$, Figure 33). Sulfate incorporation rates were similar to what was seen for proline incorporation with TGF-β1 inducing the greatest sulfate incorporation rates ($p<0.0060$) and all media groups significantly different from each other ($p<0.0060$). Sulfate incorporation rates increased through day 4 with rates higher than for all other days ($p<0.0001$) except for day 14 ($p=0.11$). Similar to what was found for proline incorporation rates, the increases in sulfate incorporation appeared to level off after day 4 ($p>0.11$). It also appeared that the same concentration of IGF-I had a muted effect on sulfate incorporation rates of fibrochondrocytes in agarose gels compared to the rates of fibrochondrocytes in native tissue (Figure 27).

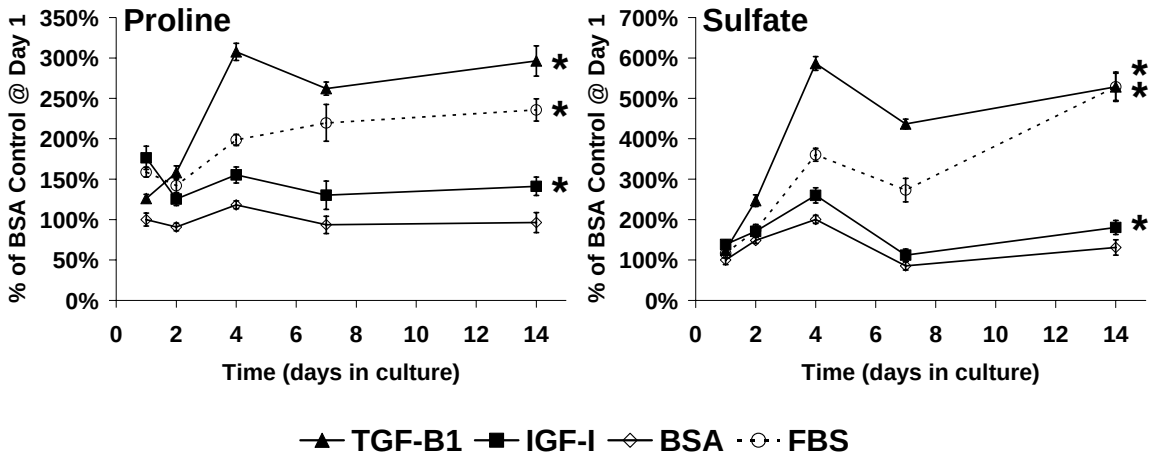

Figure 33: Time-course results for proline and sulfate incorporation rates of fibrochondrocytes in agarose gels over the two week culture period. * indicates significant difference from BSA controls ($p<0.0019$). Additionally, for both proline and sulfate incorporation rates each media condition incorporated significantly different rates from every other condition ($p<0.0060$). For presentation purposes, data are normalized to baseline BSA control values at day 1. [n=8 per media condition per time-point]

The effects of the same media conditions on articular chondrocytes in agarose gels are shown in Figure 34. The addition of FBS had the greatest effect on increasing both proline and sulfate incorporation rates of chondrocytes over all other media conditions ($p<0.0001$). Similar to the fibrochondrocytes, all media conditions significantly increased chondrocyte proline incorporation rates over BSA controls ($p<0.0001$). There were no significant differences in proline incorporation rates between TGF-β1 and IGF-I supplementation ($p=0.19$). Incorporation rates reached a steady rate after day 4 with no significant changes in proline incorporation rates after day 4 ($p>0.70$). Chondrocyte sulfate incorporation was also increased significantly by all media conditions over BSA controls ($p<0.0001$). TGF-β1 induced greater and sustained increases in sulfate incorporation rates over the IGF-I groups ($p<0.0001$). The time-course showed a short plateau in sulfate incorporation between days 4 and 7 ($p=1.0$) followed by a decline at the final time-point (day 14 less than day 7, $p=0.0077$), strongly influenced by the FBS incorporation rates. Additionally, stimulation of both proline and sulfate incorporation for the IGF-I groups was transient, showing a significant peak at day 4 ($p<0.0006$) followed by a steady decline returning to basal levels of incorporation by day 14.

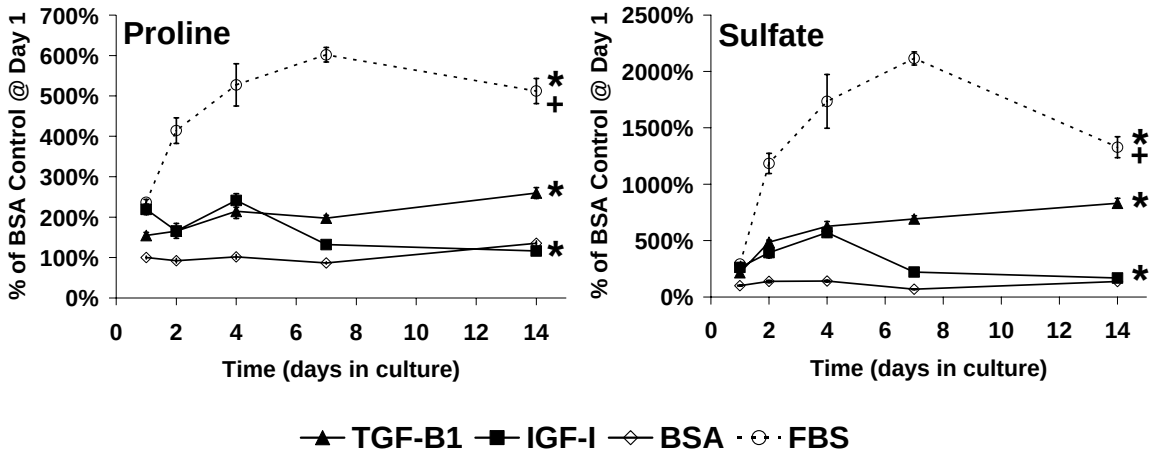

Figure 34: Time-course results for proline and sulfate incorporation rates of chondrocytes in agarose gels over the two week culture period. * indicates significant difference from BSA controls ($p<0.0001$). + indicates significant difference from TGF-β1 and IGF-I media conditions ($p<0.0001$). For presentation purposes, data are normalized to baseline BSA control values at day 1. [n=8 per media condition per time-point]

Real-Time Quantitative RT-PCR

TGF-β1 significantly increased fibrochondrocyte expression of all genes over the two-week period compared to BSA control levels (Figure 35): collagen type II ($p=0.001$), aggrecan ($p=0.013$), and collagen type I ($p=0.020$). Increased expression of both collagen types II and I occurred during the first week of culture, with no significant changes in expression from day 7 to day 14 ($p>0.16$). Aggrecan expression was significantly increased at day 1 of culture over BSA controls and remained elevated with no significant changes in expression over the two-week time period ($p=0.085$). Overall IGF-I did not induce gene expression profiles that differed significantly from those of the BSA controls for any gene studied ($p>0.31$). Fibrochondrocyte expression of collagen types II and I did significantly change with time in the IGF-I group, with a steady

increase of collagen type II expression ($p<0.0096$) and a peak in collagen type I expression at day 7 ($p<0.032$).

In general, IGF-I significantly increased chondrocyte expression of all genes over the two-week period compared to BSA control levels: collagen type II ($p=0.0001$), aggrecan ($p=0.020$), and collagen type I ($p<0.0001$). Expression of collagen type II at day 14 was significantly increased over day 1 expression ($p=0.023$). Similar to the effect of TGF-β1 on fibrochondrocyte expression of aggrecan, IGF-I significantly increased chondrocyte expression of aggrecan over BSA controls at day 1 with expression remaining elevated through day 14 ($p=0.20$). Collagen type I expression increased significantly over the first week of culture ($p=0.0076$) with no significant changes in expression from day 7 to day 14 ($p=0.83$). The addition of TGF-β1 did not significantly change chondrocyte gene expression levels relative to BSA controls for any of the genes studied ($p>0.15$). The levels of expression due to IGF-I exposure over the two-week time period did not vary significantly for any of the genes studied ($p>0.065$).

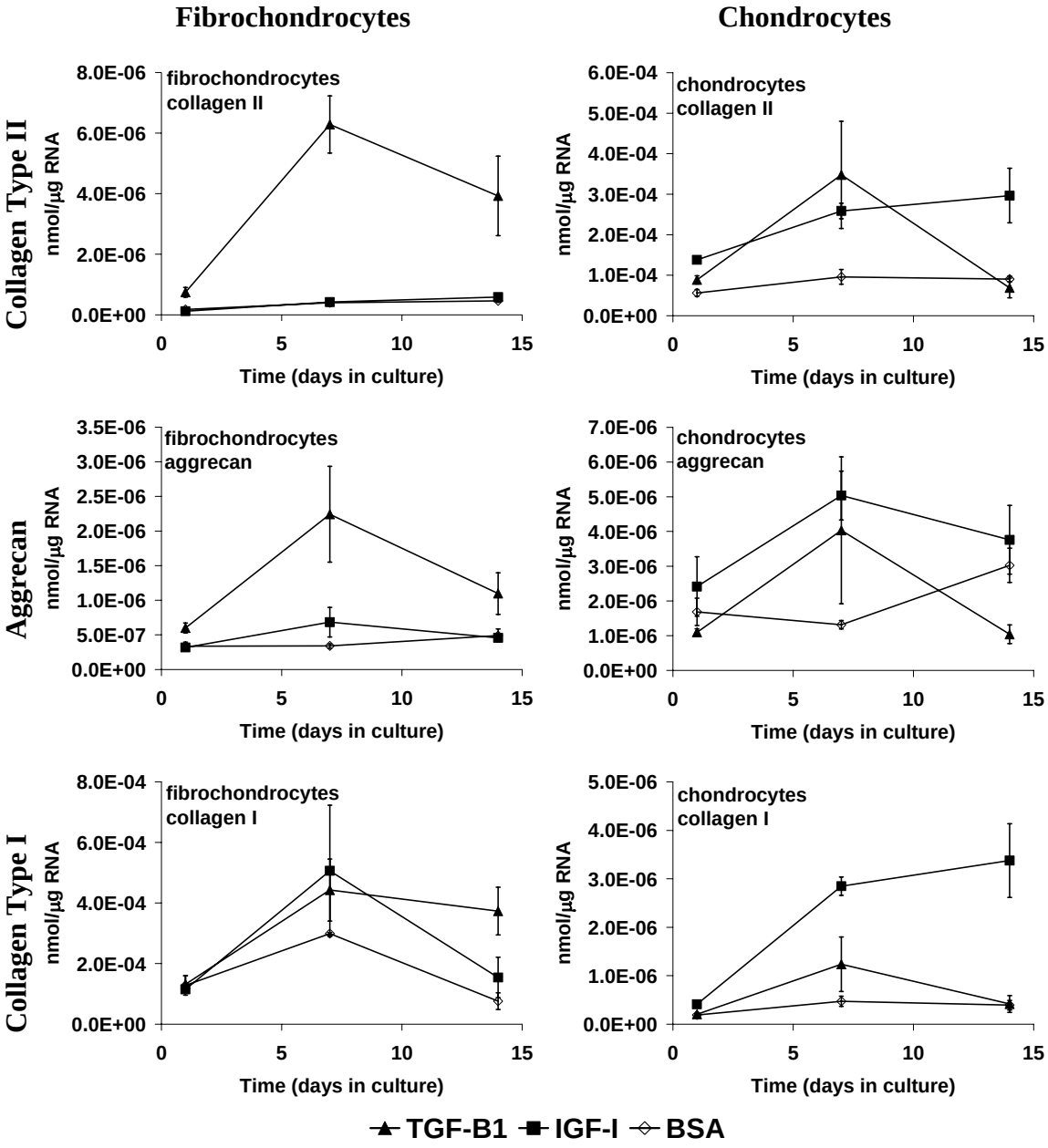

Figure 35: Changes in gene expression of collagen type II, aggrecan, and collagen type I by fibrochondrocytes and chondrocytes in agarose gel culture. Gels were cultured for up to two weeks in basal/serum-free medium (BSA) or supplemented with either 5 ng/mL of TGF-β1 or 200 ng/mL IGF-I. [n=3 per media condition per time-point]

5.4 DISCUSSION

External biochemical stimuli were shown to modulate the biosynthetic responses of meniscal fibrochondrocytes in native explant culture and agarose gel culture. Specifically, TGF-β1 was found to be the most potent stimulator of both protein and proteoglycan matrix synthesis in both culture environments. Supplementation with growth factors during two-week culture duration caused different profiles in stimulation of both proline and sulfate incorporation between tissue explants and agarose gels. Finally, at the chosen concentrations, TGF-β1 and IGF-I affected the matrix synthesis and accumulation differently when added to agarose gels seeded with either fibrochondrocytes or chondrocytes.

Although many studies have characterized levels of these factors within the articular cartilage extracellular matrix and synovial fluid, little similar information for the meniscus (or fibrocartilage in general) is currently available. Luyten *et al.* estimated 50 ng of IGF-I per gram (wet mass) of bovine articular cartilage[7]. This value is on the same order of magnitude as the concentrations shown to cause a plateau in matrix stimulation of articular chondrocytes[11,80,82]. Schneiderman *et al.* found a lower concentration of 10 ng total IGF-I per gram of adult human articular cartilage[83]. bFGF has been found in articular cartilage at concentrations of 1-50 ng per gram of tissue[11]. PDGF has been identified as a locally produced factor, with no traces of the growth factor in the circulation[128]. However, in a study exploring the use of meniscal rasping as a technique to promote healing within the avascular zone, Ochi *et al.* detected the induction of the expression of both TGF-β1 and PDGF using immunohistochemical staining[84]. These

factors were thought to play an important role in the recruitment of fibrochondrocytes and synoviocytes to commence the repair process.

It should be noted that the choice of control conditions (specifically, serum supplemented vs. defined media) may confound comparisons between studies. The use of a serum-supplemented control increases the baseline synthesis rates. For example, the stimulation of meniscus proteoglycan synthesis by TGF-β1 in the current study was substantially greater than that previously reported by Collier and Ghosh for mature ovine meniscus tissue, who found a twofold increase over 10% fetal bovine serum control levels with the addition of 4 ng/mL TGF-β1[40]. This is in contrast to a sixfold to sevenfold increase at 5 ng/mL TGF-β1 for bovine meniscus explants in the current study and a threefold increase at 3 ng/mL for porcine meniscus explants reported by Lietman *et al.* (both over BSA controls)[129].

In addition to serum-supplemented controls, many studies have added their cytokines into a serum-supplemented environment. Serum from the manufacturer is usually not characterized for individual cytokines or binding proteins, both of which may interact with the action of the target cytokine. These studies introduced their target cytokines to a diverse, uncharacterized milieu of cytokines (anabolic and catabolic in nature) that has the ability to interact with the target cytokines, again confounding the ability for comparison between studies. A recent set of studies from Tumia and Johnstone investigated the effects of either bFGF or IGF-I on fibrochondrocyte proliferation and matrix synthesis in monolayer in the presence or absence of FBS[130,131]. They found a greater general fibrochondrocyte response to bFGF in the serum-free media group as compared to the serum-supplemented group. Responses to IGF-I were varied

between serum-free and serum-supplemented groups, suggesting interplay between bFGF and IGF-I supplementation and other factors in the serum.

In contrast to the response of articular cartilage, the relative stimulation of meniscus sulfate incorporation by TGF-β1 was even more robust than that previously reported for articular cartilage. Under culture conditions similar to ours, Morales and Roberts found that stimulation of immature bovine articular cartilage proteoglycan synthesis reached a plateau by 10 ng/mL of TGF-β1 with a sevenfold to eightfold increase over BSA controls[79]. In contrast, the current dose-response study on meniscus explants indicated a monotonic increase in sulfate incorporation for doses up to 100 ng/mL of TGF-β1, reaching a tenfold increase over BSA controls (to approximately the level of cartilage explants in basal medium) with no indication of a plateau in the response. This, along with previous evidence that TGF-β1 stimulation of fibrochondrocytes preferentially stimulates the production of large, aggregating proteoglycans[40], suggests that high doses of TGF-β1 induce the production of a more cartilaginous matrix.

Focusing on the mitogenic response, Spindler *et al.* reported that PDGF-AB induced a tenfold increase in proliferation by adult ovine explants from the outer one-third but no significant change in proliferation by explants from the inner two-thirds, even with PDGF-AB supplementation at 200 ng/mL[58]. They suggested that the fibrochondrocytes of the central region may not have the α-PDGF or β-PDGF receptors necessary to interact with the exogenous PDGF-AB. In the current study, neither PDGF-AB nor any other factor significantly increased explant DNA content (data not shown). However, PDGF-AB did modulate the biosynthesis of tissue explants from the

midsubstance of bovine menisci, suggesting that immature bovine fibrochondrocytes do have functional PDGF receptors. This is consistent with previous findings that PDGF stimulation of both immature and mature bovine articular cartilage had no influence on DNA synthesis but induced a dose-dependent increase in proteoglycan biosynthesis[132]. It is also consistent with the dose-response studies of Lietman *et al.* showing a dose-dependent increase in proteoglycan biosynthesis of porcine meniscus expants[129].

The actions of bFGF on articular chondrocytes have been identified as both mitogenic[80,81] and biosynthetic[11,80]. Osborn *et al.* found saturation in proteoglycan synthesis at 100-1000 ng/mL for adult bovine articular chondrocytes[80], with [^{3}H]-thymidine incorporation also significantly stimulated at the highest concentrations of bFGF. Sah *et al.* found consistent results for adult bovine articular cartilage with bFGF supplementation[11], but found that matrix production by immature bovine cartilage was stimulated only at a concentration of 3 ng/mL of bFGF. Concentrations of 30-300 ng/mL of bFGF caused an inhibition of both matrix production and mitogenic activity and induced catabolism with increases in proteoglycan release from the tissue matrix. In contrast, we observed a significant stimulation of proteoglycan accumulation by immature meniscus explants with up to 300 ng/mL of bFGF, indicating that the two tissues may respond in different manners to specific stimuli.

Studies on articular cartilage explants have found plateaus in IGF-I stimulation of proteoglycan synthesis from 20-200 ng/mL[7,11,80,82]. This wide range of concentrations has been attributed to differences in immature versus mature tissue[11,80], where mature tissue requires lower concentrations of IGF-I for maximal responses in proteoglycan production. Our results for immature fibrocartilage are on the same order of magnitude of

the upper end of that concentration range. While observing stimulation of proteoglycan production in response to IGF-I, Luyten *et al.* also found no change in DNA content over a 5 week culture period with IGF-I supplementation of immature (newborn to 10 month olds) articular cartilage explants[7]. Our results were similar, with no significant change in DNA content normalized to explant dry weight over the 2 week culture period (data not shown).

In the current agarose gel studies the effects of growth factor supplementation on gel maturation by fibrochondrocytes or chondrocytes with either 5 ng/mL TGF-β1 or 200 ng/mL IGF-I (same concentrations used for tissue explants) were studied. Additionally, 10% FBS was used as an additional control group. There were striking differences between the behaviors of the fibrochondrocyte and chondrocytes in agarose gel culture. As there is currently only a single study in the literature that has characterized the behavior of fibrochondrocytes in agarose gel culture[57], these results will complement the characterization of fibrochondrocytes in a 3-D culture as well as the existing chondrocyte/agarose studies. Again for analysis and comparison of these studies to others, the choice of control culture medium and supplemented media formulae are important issues to consider.

The actions of these growth factors have the potential to elicit a greater response in the agarose gel cultures over what was seen in the tissue explant studies. With the exception of a muted effect of IGF-I on sulfate incorporation rates, the fibrochondrocytes in agarose gels had increased and sustained matrix synthesis rates. One reason for this could be the increased accessibility of the growth factors to the cells in the agarose gel culture system due to considerably higher diffusivity and likely lower binding protein

concentrations. Similar to the free swelling *in vitro* culture of these studies, fibrochondrocytes and chondrocytes *in vivo* rely upon diffusion and convection through the tissue matrices for delivery of their nutrients and factors such as cytokines from the synovial environment. The ability of these factors to attach to a cellular receptor is strongly influenced by the concentrations and affinities of appropriate binding proteins, particularly in the cases of IGF-I and members of the TGF-β superfamily (including TGF-β1). Both IGF-I[133,134] and TGF-β1[128,135] have binding proteins that have high affinities for each factor and can act to "trap" the growth factors within the extracellular matrix, modulating growth factor availability for cellular receptor binding. These binding proteins exist in the native tissue explants from the outset of culture, however in the agarose gel culture the presence of these proteins is dependent on cellular production. Additionally, the IGF-I binding proteins can exist in a soluble form, complexing with IGF-I they form larger molecules (~160 kDa) in the synovium that have increased difficulty of penetrating the articular cartilage matrix[83,133].

Although extensive work had been done on chondrocytes in agarose gel culture, Buschmann *et al.* were the first to use agarose as a scaffold to produce a "mechanically functional cartilage-like matrix"[87]. This study used immature bovine chondrocytes seeded in 2% agarose at a high seeding density of ~1–2x10^7 cells/mL. The gels were cultured in medium supplemented with 10% FBS and assessed for biochemical composition, biosynthesis, and material properties at various times during the 70 day culture. At 35 days of culture biochemical content, stiffness, and streaming potential had reached 25% those of immature articular cartilage. Histologically, the chondrocytes had

developed a uniform matrix composed of collagen and proteoglycan, concentrated pericellularly at first and extending into the interterritorial space with maturation.

Mauck *et al.* extended these results to look at the effects of the addition of either TGF-β1 at 10 ng/mL or IGF-I at 300 ng/mL to serum-supplemented medium on the maturation of 2% agarose gels seeded with $1x10^7$ cells/mL over 35 days of culture[16]. They also looked at the superposition of oscillatory mechanical compression on gel maturation (to be discussed in Chapter 6). The addition of either factor significantly increased sGAG deposition 2-fold over serum-only control samples with accelerated rates of deposition during the first week of culture. In contrast, collagen deposition was not significant until day 28 of culture, where the rates increased sharply through the end of culture. At this point both factors increased collagen deposition by 6- and 4-fold for TGF-β1 and IGF-I, respectively. As this study is the closest study looking at similar contributions of growth factors on matrix deposition, it is important to note that the growth factors were added to a serum-supplemented environment. They were looking at additive affects of the growth factors for a functional cartilage tissue engineering approach, not to explore the individual effects of each factor, which is what the current studies were focusing on.

In the current study looking at gel maturation over 14 days, there was no increase in DNA content with the addition of either TGF-β1 or IGF-I for either cell type. TGF-β1 and IGF-I have been shown to induce proliferation of chondrocytes[9,136,137] and fibrochondrocytes in monolayer[130]. TGF-β1 has been shown to have no effect on DNA synthesis of chondrocytes in explant culture[79,138]. In contrast, IGF-I did not change DNA content of immature cartilage explants[7,11], but did increase the DNA content of adult

explants[11]. The effects of TGF-β1 on chondrocytes also depend highly upon which part of the cell cycle the chondrocyte is in. Vivien *et al.* have shown that chondrocytes in the S-phase (DNA actively synthesized) of the cell cycle exhibit an increased number of high-affinity TGF-β1 binding sites as compared to quiescent cells in the G0 or G1 phases[139]. Due to the increased matrix deposition of the fibrochondrocytes and chondrocytes, the cells were likely shifted towards matrix synthesis processes over proliferative processes, and may have been expressing lower affinity TGF-β1 receptors because of their cell cycle state.

As previously discussed, both factors have been shown to increase matrix synthesis; mainly increases in proteoglycan were measured. In comparing the matrix synthesis of the two cell types, the FBS media condition consistently enhanced matrix deposition over the basal/serum-free (BSA) media condition. Interestingly, accumulation of sGAG in the fibrochondrocyte seeded gels was enhanced the greatest with the addition of TGF-β1, with accumulation greater than the FBS group. This was not the case for the chondrocytes, where the addition of TGF-β1 at 5 ng/mL caused increased sGAG accumulation that was only greater than the BSA control condition. These findings suggest that: [1] it is not the TGF-β1 alone that is responsible for stimulation of proteoglycan production by chondrocytes; it is a combination of anabolic factors present in the serum that stimulates the cells or [2] that the FBS contains a much higher concentration of TGF-β1 that is able to significantly stimulate sGAG synthesis or [3] even though we are using similar concentrations to what is in the literature, the actual "equivalent" doses (taking into account differences in cytokine availabilities among monolayer versus gels versus tissue explants) are not the same. Although the second

proposal has not been quantified for the serum lot used, it is likely that this is not the contributor of increased sGAG stimulation. One reason supporting this argument is that even if TGF-β1 is at higher concentrations than 5 ng/mL in the serum it is likely in a latent form and complexed to a latency-associated peptide. Enzymatic activity or extreme pH values are necessary for the TGF-β1 to be released and activated, available for cellular binding[127].

When looking at the amount of unincorporated matrix components expelled into the surrounding media, for both cell types, supplementation with FBS caused the greatest amount of both sGAG and collagen into the media. This shows that either [1] the FBS is causing the greatest amount of matrix synthesis of both cell types such that the matrix components are getting produced faster than they can be assembled into the existing matrix or [2] catabolic cytokines are present (either in FBS or produced by the cells), causing turnover of accumulated matrix components or [3] a combination of these things. Again, the content of cytokines in the serum or spent media was not quantified; therefore a strong conclusion cannot be reached.

The levels of L-5-^{3}H-proline and ^{35}S-sodium sulfate incorporation as measures of total protein and proteoglycan synthesis also varied between cell types. In general, the relative sulfate incorporation rates among the four media conditions were consistent with the sGAG accumulation curves for both cell types. TGF-β1 stimulated the greatest increases in proline and sulfate incorporation rates for fibrochondrocytes that were sustained over the 14 day culture period. As seen in the tissue explants, both TGF-β1 and IGF-I caused greater increases in sulfate incorporation rates than in proline incorporation rates. Proline incorporation rates appeared to level off after 4 days of culture for all

media conditions, suggesting a sustained response of the cells to their media environment. This is in contrast to what was seen for proline incorporation in the tissue explants with no difference in incorporation between media treatments. This difference could be attributed to differences in cell-matrix interactions within the gels and native tissue. As an example, it has been shown that the response of chondrocytes to TGF-β1 exposure relies upon cellular binding to collagen type II via the β1 integrin[140]. Since the cell-matrix interactions are potentially different (molecule quantity, organization, etc.) in the two different culture systems studied, these interactions could perhaps direct fibrochondrocyte response to the same biochemical stimuli.

Proline and sulfate incorporation rates by chondrocytes were the greatest in the FBS supplemented agarose gels. A decrease in incorporation rates were seen from day 7 to 14, especially in FBS cultures. This decrease in matrix synthesis could represent the chondrocytes transitioning from a matrix synthesis phase to a proliferative phase as indicated by the increase of day 14 DNA content in the chondrocyte seeded agarose gels. Looking at the incorporation rates of ^{3}H-thymidine would be a better indicator of cellular proliferation. This decrease in incorporation rates was also seen by Buschmann *et al.* where increased levels of incorporation were detected early in their culture supplemented with FBS and returned to levels of native tissue explants after one month in culture[87]. Morales and Roberts also saw a convergence in sulfate incorporation rates between explants cultured in 5 ng/mL of TGF-β1 and 10% FBS after 21 days of culture[79]. It appears that the current model is behaving in manners similar to what has been previously reported.

The final set of endpoints studied for the cells in agarose gels was gene expression of cartilaginous markers (collagen type II and aggrecan) and a more fibrocartilaginous marker (collagen type I). The first studies placing chondrocytes in agarose gel culture showed the support of a chondrogenic phenotype in agarose gel culture[85,141,142]. Webber *et al.* showed that on a morphological basis fibrochondrocytes were able to keep their phenotype through 28 days of culture in agarose gel culture[57]. The real-time quantitative RT-PCR results presented here represent a preliminary look at the changes in gene expression over the 14 day culture period due to growth factor supplementation. It appears that the addition of TGF-β1 pushed the fibrochondrocytes towards a more chondrocytic phenotype with increased mRNA expression of both collagen types II and aggrecan. These increases, although higher than induced expressions due to IGF-I or BSA, were still 1-2 orders of magnitude lower than expression levels of chondrocytes. Due to the limited size of the study, there was a high variability between expression levels, which makes it difficult to come to definitive conclusions about the data.

In conclusion, growth factors have been shown to modulate matrix biosynthesis of fibrochondrocytes in native tissue explants. Each growth factor studied showed dose-dependent increases in matrix synthesis with increasing concentrations. Sustained increases in proteoglycan synthesis were seen with growth factor exposure spanning two weeks. Additionally, fibrochondrocytes in agarose gel culture were stimulated to produce *de novo* matrix with the same duration of exposure. These findings show the ability of the fibrochondrocytes, often thought of as existing in a quiescent state in their native matrix, to respond to biochemical stimuli. The results indicate that the fibrochondrocytes are capable of producing native matrix components in response to

these factors, important in the consideration of regenerative and reparative technologies. However, these studies also bring about the importance of considering the knee joint as a whole when developing such technologies. Potential treatments targeted at one tissue may have detrimental effects on another tissue, as seen here with different effects of specific growth factors on matrix synthesis between chondrocytes and fibrochondrocytes.

CHAPTER 6

COMBINED STIMULATION OF MENISCAL FIBROCHONDROCTYES IN EXPLANT AND AGAROSE GEL CULTURE BY MECHANICAL LOADING AND CYTOKINE SUPPLEMENTATION

6.1 INTRODUCTION

The studies in Chapters 4 and 5 examined the independent effects of compression and cytokine supplementation. The *in vivo* environment of the soft tissues of the knee involves a complex combination of mechanical stresses and a milieu of cytokines. The mechanisms by which fibrochondrocytes and chondrocytes respond to these distinct classes of stimuli have recently received heightened awareness. Beginning to explore the mechanisms by which cells respond to each stimulus and possible interactions, if any, will aid in the understanding of the varied mechanical loads and cytokine concentrations seen during the developmental processes, normal homeostasis, and diseased states in the soft tissues of the knee.

The combined effects of mechanical loading, static compression[14], dynamic compression[15], and dynamic shear[143], and IGF-I supplementation on immature articular cartilage explants have been examined. Additionally, the mature porcine meniscal explant model has been used to explore the effects of stimulatory mechanical strains and the catabolic factors of interleukin-1[52] or tumor necrosis factor-α[144] in an effort understand changes in the meniscus that may occur in the pathological development of joint disease. The use of combinatory anabolic stimuli as a strategy for the development of *de novo* matrix in tissue engineering applications has also gained attention recently.

Mauck *et al.* have shown the synergistic effects on matrix accumulation by adding either TGF-β1 or IGF-I to an oscillatory compression protocol[16]. Gooch *et al.* have shown additive effects of chondrocyte matrix deposition on polyglycolic acid scaffolds supplemented with IGF-I while cultured in a rotating bioreactor[145].

Additionally, *in vivo* implantation of the bioactive small intestinal submucosa (SIS) scaffold to promote meniscal regeneration in a canine model has shown promise as a fibrocartilage tissue-engineering approach[146-148]. Arthroscopically implanted acellular SIS disks supported native synoviocyte infiltration, proliferation, and matrix deposition, suggestive of the development of *de novo* fibrocartilage in response to the complex *in vivo* environment. Although animal implantation is highly uncontrolled compared to *in vitro* studies, the results provide insight into remodeling events seen in a loaded joint rich in cytokines and may provide suggestions for preculture criteria.

We hypothesized that the anabolic stimulation of matrix synthesis due to growth factor supplementation would be decreased with static compression. The first studies explored the combined effects of static compression up to 50% and supplementation with growth factors on biosynthesis of meniscus tissue explants. The second set of studies explored the combined effects of static compression and growth factor supplementation on the biosynthesis and gene expression of fibrochondrocytes and chondrocytes in agarose gel culture. We also hypothesized that the superposition of oscillatory compression on samples supplemented with growth factors would have greater than additive effects on matrix synthesis. These studies explored the addition of oscillatory compression at 1.0 Hz to agarose gels seeded with either fibrochondrocytes or chondrocytes and grown in the presence of a specific growth factor.

6.2 MATERIALS AND METHODS

6.2.1 Tissue and Cell Harvest Procedure

Meniscus tissue explants (4 mm diameter x 2 mm thick) and 3% agarose gels (6 mm diameter x 3 mm thick) seeded with fibrochondrocytes or articular chondrocytes at 5 x 10^6 cells/mL were used for these studies. Tissue and cell isolation procedures are detailed in Chapter 3. Explants and agarose gels were precultured in basal/serum-free medium (DMEM plus 0.1% BSA, 0.1 mM NEAA, 1.0 mM HEPES, 50 μg/mL gentamicin, 0.25 μg/mL fungizone, 0.4 mM L-proline and 50 μg/mL ascorbate) for 3 days following excision or casting.

6.2.2 Static Compression

Tissue explants

These studies looked at effects on matrix synthesis of static compression combined with growth factor supplementation (Table 5): bFGF at 100 ng/mL (5.81 nM), IGF-I at 100 ng/mL (13.2 nM), PDGF-AB at 100 ng/mL (3.92 nM), or TGF-β1 at 50 ng/mL (2.0 nM). After preculture, tissue explants (n=6 per compression level per growth factor) were cultured for 4 days in basal media with or without a single concentration of growth factor under static compression to 100%, 75% or 50% of the original cut thickness (hereafter referred to as 0%, 25%, or 50% compression, respectively). Tissue explants were compressed within the custom designed polycarbonate static compression chambers described in Chapter 4. Two sub-studies were conducted using tissue from two different animals. During the preculture period, the explants swelled such that

compression to the original cut thickness imparted a slight compressive deformation on the samples. Control free swell (FS) explants were cultured 48-well plates.

Table 5: Cytokine concentrations used for the compression and cytokine interaction studies. Tissue explants and agarose gels were precultured for 3 days in basal/serum-free (BSA) media. Following preculture, the explants or gels were randomly assigned to a media group containing the concentrations of individual growth factors shown below.

	Concentrations (ng/mL)	
Growth Factor	Tissue explants	Agarose gels
bFGF	100	*n/a*
IGF-1	200	200
PDGF-AB	100	*n/a*
TGF-β1	5	5

Agarose gels

Agarose gels were statically compressed in the compression chambers designed for the oscillatory loading system presented in Chapter 4 and detailed in Appendix A. A subset of the cytokines was chosen for the gel studies. TGF-β1 and IGF-I were used at concentrations matching those used for the explant studies (Table 5). After preculture in basal medium, the gels were allowed to culture for 7 days in one of three media conditions (n=8 per media condition per compression level per cell type): basal medium + 5 ng/mL TGF-β1, basal medium + 200 ng/mL IGF-I, or basal medium alone. Gels were also cultured in a fourth media condition containing 10% FBS (DMEM plus 10% FBSA, 0.1 mM NEAA, 1.0 mM HEPES, 50 μg/mL gentamicin, 0.25 μg/mL fungizone,

0.4 mM L-proline and 50 μg/mL ascorbate). The gels were placed under static compression of 0%, 10%, 25%, or 50% or maintained free swelling (FS) during the final 21 hours of culture. Radiolabeled precursors at concentrations of 10 μCi/mL of L-5-^{3}H-proline and 5 μCi/mL of ^{35}S-sodium sulfate were added to the respective media formulations during the compression.

A subset of the agarose gels (n=4 per media condition per compression level per cell type) for the static compression studies were separated into "ring" and "center" samples to probe for transport limitations within the gels. A 4 mm diameter biopsy punch was used to obtain the sub-samples, and the samples were then digested separately. On a volume basis, the ring accounts for approximately 56% of the total gel volume (total volume is approximately 84 μL) and the center accounts for the remaining 44%.

Real-Time Quantitative RT-PCR

A separate study was performed using agarose gels seeded with either articular chondrocytes or fibrochondrocytes at an increased cell density of 20 x 10^{6} cells/mL. A sufficient amount of RNA (>0.25 μg) for analysis was obtained from each increased cell density gel. The gels were prepared, precultured, and loaded in the same manner as previously described. Following static compressive loading, the samples were immediately placed in 600 μL of RLT lysis buffer and melted at 60°C for 10 minutes. Samples were then place in the -80°C freezer for subsequent RNA isolation (see Chapter 3 for details). Gene expression for collagen types I and II and aggrecan were examined to compare the free swelling control gels to the 50% compression groups when cultured under one of three media conditions: basal medium + 5 ng/mL TGF-β1, basal medium + 200 ng/mL IGF-I, or basal/serum-free medium alone.

6.2.3 Oscillatory Compression

The combined effects of intermittent oscillatory compression and cytokine supplementation on chondrocytes and fibrochondrocytes in agarose gels were explored in this study. After preculture in basal/serum-free medium containing 0.1% BSA, the gels were allowed to culture for 7 days in one of four media conditions (n=8 per media condition per compression condition per cell type): basal medium + 5 ng/mL TGF-β1, basal medium + 200 ng/mL IGF-I, basal medium alone, or 10% FBS. The gels were then subjected to the intermittent oscillatory compression protocol described in Chapter 4 (2 x [12-hours 10% ± 3% at 1.0 Hz followed by 12-hours 10% static offset]) during the 7^{th} and 8^{th} days of culture. Control gels were held at a static offset of 10% compression. Radiolabeled precursors at concentrations of 10 μCi/mL of L-5-^{3}H-proline and 5 μCi/mL of ^{35}S-sodium sulfate were added to the respective media formulations for the duration of loading.

All of the agarose gels for the oscillatory compression studies were separated into "ring" and "center" samples to probe for transport and fluid flow limitations within the gels. A 4 mm diameter biopsy punch was used to obtain the sub-samples, and the samples were then digested and analyzed separately using previously described techniques.

6.3 RESULTS

6.3.1 Static Compression

Tissue explants

In the presence of any one growth factor, both the normalized sulfate and proline incorporation rates of fibrochondrocytes in tissue explants were significantly inhibited for all static compression levels as compared to the free swell groups ($p<0.0001$, Figure 36). Increasing compression levels induced a significant dose-dependent inhibition of proline incorporation ($p<0.0001$), but not of sulfate incorporation ($p>0.48$). Interestingly, while the overall levels of sulfate incorporation varied substantially among media conditions, the dose-dependent inhibition of proline incorporation relative to free swell incorporation levels did not vary significantly among media conditions ($p=0.086$), although this would have to be verified with a larger sample size. Since the samples swelled during the preculture period, explants in the 0% compression group were actually compressed somewhat, resulting in significant inhibitions of sulfate and proline incorporation rates relative to free swell controls for all media conditions ($p<0.0001$). Although the swelling was not quantified, this phenomenon is consistent with previous observations in our laboratory[149].

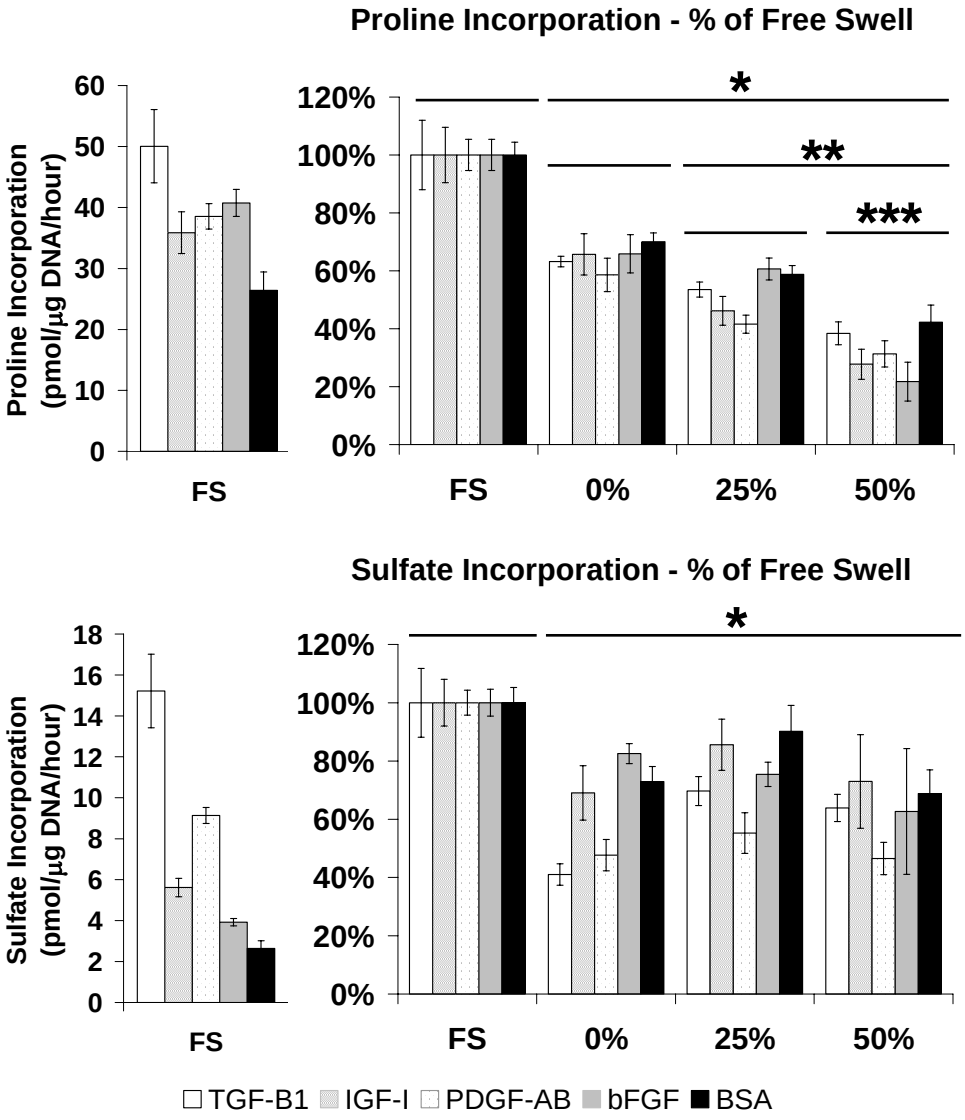

Figure 36: Effects of static compression on proline and sulfate incorporation rates of meniscus tissue explants for TGF-β1, IGF-I, PDGF-AB, or bFGF supplemented media conditions. Results for compression groups are normalized to the average of the corresponding media's free swell (FS) group (shown to the left). * indicates significant difference from free swell ($p<0.0001$). ** indicates significant difference from 0% compression ($p<0.0001$). *** indicates significant difference from 25% ($p<0.0001$). [n=6 per media condition per compression level]

Agarose gels

Fibrochondrocytes

The data presented for the fibrochondrocytes in agarose gels represents pooled data from a sub-set of gels that were analyzed as wholes (n=4) and the other sub-set that were separated into rings and centers and analyzed individually. In the statistical analysis of the incorporation rates relative to the free swell controls (FS), two data points in the IGF media condition at 10% compression were identified as outliers with greatly increased incorporation values lying outside 2.5 standard deviations from the mean. Therefore, the sample size for this data point was 6 as compared to the balance of the conditions with sample sizes of 8.

In the presence of any one growth factor, both the normalized proline and sulfate incorporation rates of fibrochondrocytes in agarose were significantly inhibited only when placed under 50% static compression as compared to free swelling controls for any media condition ($p<0.0001$, Figure 37). Agarose gels held at 50% compression exhibited both proline and sulfate incorporation rates that were significantly lower than the collective rates of any other compression percentage ($p<0.031$ for proline and $p<0.042$ for sulfate). While the non-normalized sulfate incorporation rates varied substantially among media conditions, the inhibition of sulfate incorporation relative to free swell incorporation levels did not vary significantly among media conditions ($p=0.82$). In contrast, there were differences in the relative inhibitions of proline incorporation rates among the four media conditions. Fibrochondrocytes in the TGF-β1 or FBS media conditions had greater levels of inhibition in proline incorporation rates relative to free swell controls than cells in either BSA or IGF media conditions ($p<0.035$).

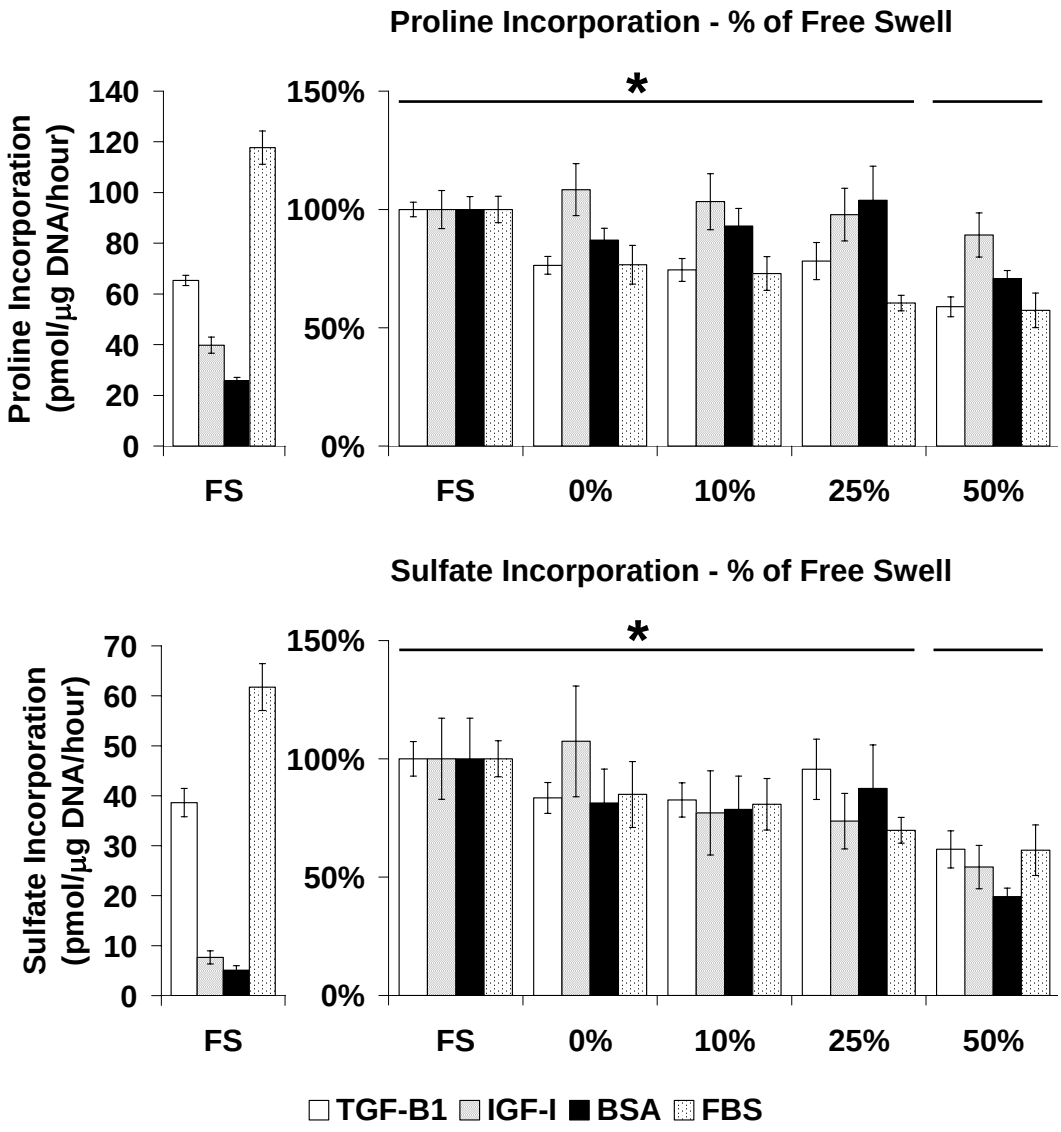

Figure 37: Effects of static compression on proline and sulfate incorporation rates of fibrochondrocyte seeded agarose gels. Results for compression groups are normalized to the average of the corresponding media's free swell (FS) group (shown to the left). * indicates significant difference from 50% ($p<0.042$). [n=8 per media condition per compression level, n=6 for 10%, IGF-I group]

Articular Chondrocytes

The data presented for the chondrocytes in agarose gels represents data from a sub-set of gels that were analyzed as wholes (n=4) only. The sub-set of gels that were separated into rings and centers had been erroneously assigned to different media and compression groups during the analysis portion of the study, giving inconsistent results. Therefore, the data for the "halved" gels will only be presented when analyzing differences in ring and core matrix synthesis as the rings and their corresponding centers were not affected by the reordering of the samples.

In the presence of any one growth factor, both the normalized proline and sulfate incorporation rates of chondrocytes in agarose were significantly inhibited when placed under 50% compression as compared to all other mechanical environments ($p<0.0087$, Figure 38), including the free swell control condition. Samples held at 0% and 25% compression had increased levels of normalized proline incorporation rates over the free swell controls ($p<0.017$) and increased levels of normalized sulfate incorporation rates over samples held at 25% compression ($p<0.027$). Samples held at 0% compression also had increased levels of sulfate incorporation relative to free swell controls ($p=0.0053$) and relative changes in proline incorporation rates compared to samples held at 25% compression ($p=0.036$). There was a slight significant difference seen in the normalized proline incorporation rates between media conditions with the IGF-I media condition having greater changes in incorporation rates relative to free swell controls than the changes for the BSA media condition ($p=0.0083$). In contrast, the changes in sulfate incorporation rates relative to free swell controls were significantly different between all media conditions ($p<0.0001$).

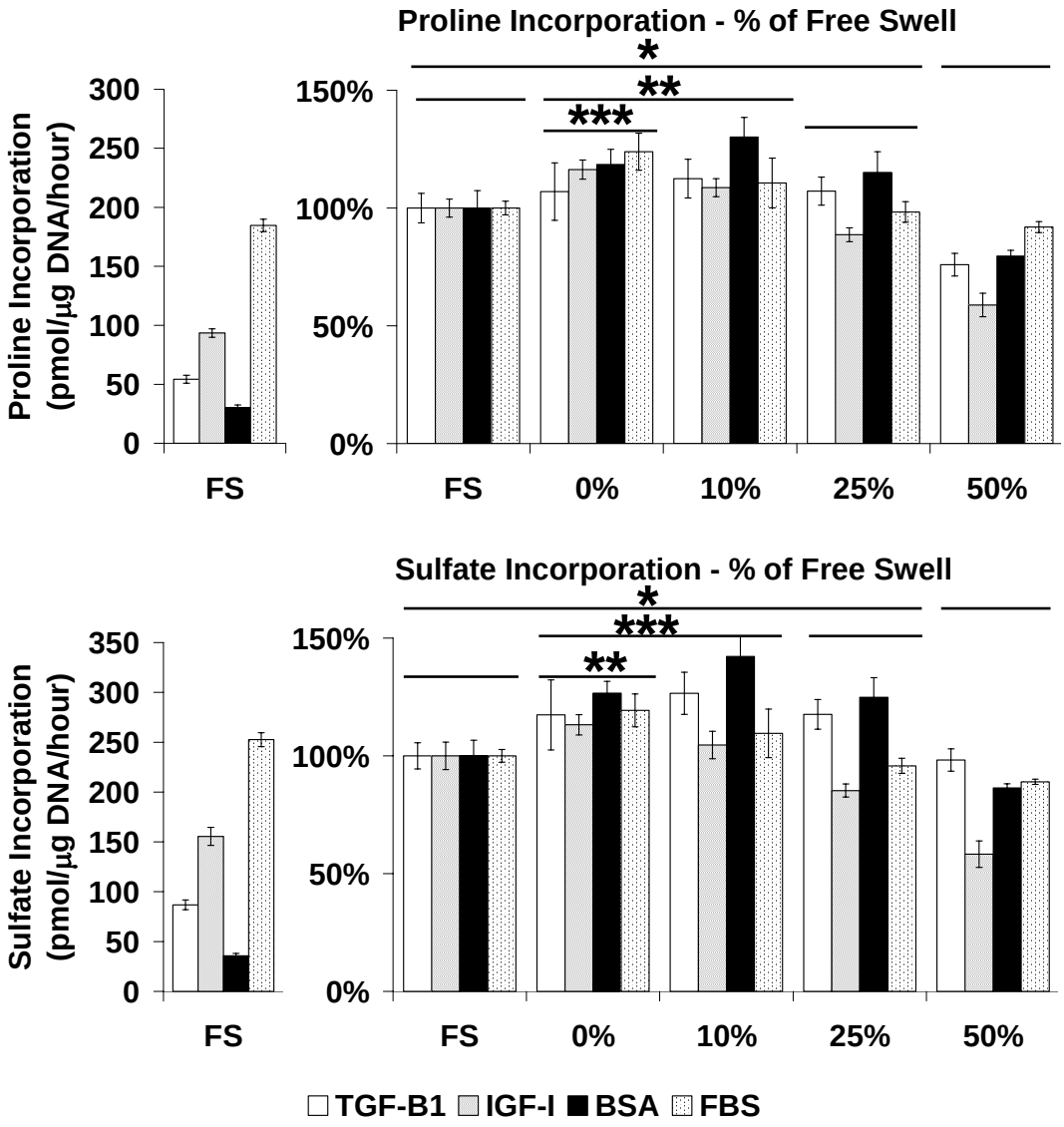

Figure 38: Effects of static compression on proline and sulfate incorporation rates of chondrocyte seeded agarose gels. Results for compression groups are normalized to the average of the corresponding media's free swell (FS) group (shown to the left). * indicates significant difference from 50% ($p<0.0087$). ** indicates significant difference from free swell ($p<0.017$). *** indicates significant difference from 25% ($p<0.036$). [n=4 per media condition per compression level]

Ring vs. Center

The sub-set of samples that were divided into rings and centers were analyzed for differences in incorporation rates. Incorporation rates normalized to DNA content (to account for discrepancies in volume between ring and center) for proline and sulfate were pooled to obtain average incorporation rates for rings and centers for all media conditions and all compression conditions. The data shown in Figure 39 depict the ratio of the ring incorporation rates normalized by the center incorporation rates. Significantly higher sulfate incorporation rates were found in the rings of the agarose gels seeded with fibrochondrocytes ($p<0.0024$). It can be noted, however, that this difference was due to the striking regional discrepancies in the agarose gels in the FBS media condition. In this condition, gel rings had incorporation rates of both proline and sulfate that were significantly higher than gel centers ($p=0.0016$ and $p<0.0001$, respectively). When the FBS media condition was taken out of the pooled data, both incorporation rates were not significantly different between rings and centers (data not shown, $p>0.46$). This was the case for the pooled data of the agarose gels seeded with articular chondrocytes ($p>0.40$). Due to the digest solution volume used for these studies, the balance of these samples was not sufficient for biochemical content, specifically sulfated glycosaminoglycan quantification. However, in the following oscillatory compression study, biochemical content was determined for ring and center gel portions.

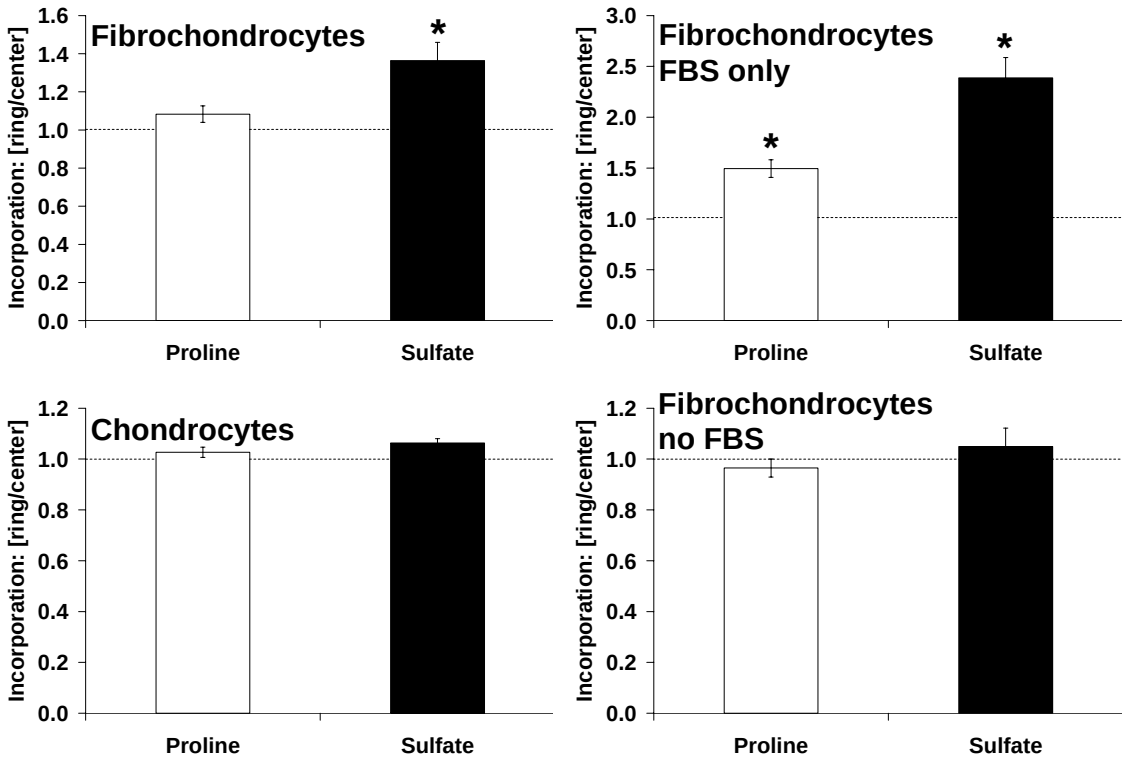

Figure 39: Incorporation rates of proline and sulfate for 6 mm diameter outer rings of the agarose gels normalized by the 4 mm diameter centers. Data represents pooled results from all 4 media conditions and all 5 loading conditions. * indicates significant difference between ring and center ($p<0.0024$). [n=80, n=20 for FBS only, n=60 for no FBS]

Real-Time Quantitative RT-PCR

The gene expression data for both fibrochondrocytes and chondrocytes seeded in agarose gels were found to be non-normal. To confer normality to the data set, a Box-Cox transformation was performed on expression levels of each gene by each cell type using the Minitab calculated optimum lambda coefficient. The transformed data were put into a General Linear Model using a two-factor analysis with interaction term (media, compression condition, and interactions between media and compression condition). For all levels of gene expression for both cell types, the interaction term was non-significant ($p>0.065$), indicating independence of media and compression effects.

For all genes studied, fibrochondrocyte expression levels were significantly downregulated by 50% static compression relative to free swelling controls ($p<0.0008$, Figure 40). There was also a downregulation of aggrecan and collagen type I gene expression for the chondrocytes in response to 50% compression ($p<0.030$). However, there was not a significant effect of compression on the regulation of collagen type II expression ($p=0.24$). There were no significant differences due to media condition in expression levels of collagen type II and aggrecan for the fibrochondrocytes. IGF-I induced the greatest expression levels of collagen type I by the fibrochondrocytes ($p<0.0005$). Culture with either TGF-β1 or IGF-I significantly upregulated both collagen type I and II expression levels relative to BSA controls ($p<0.014$). Finally, chondrocyte expression of aggrecan was significantly upregulated relative to BSA controls ($p=0.018$).

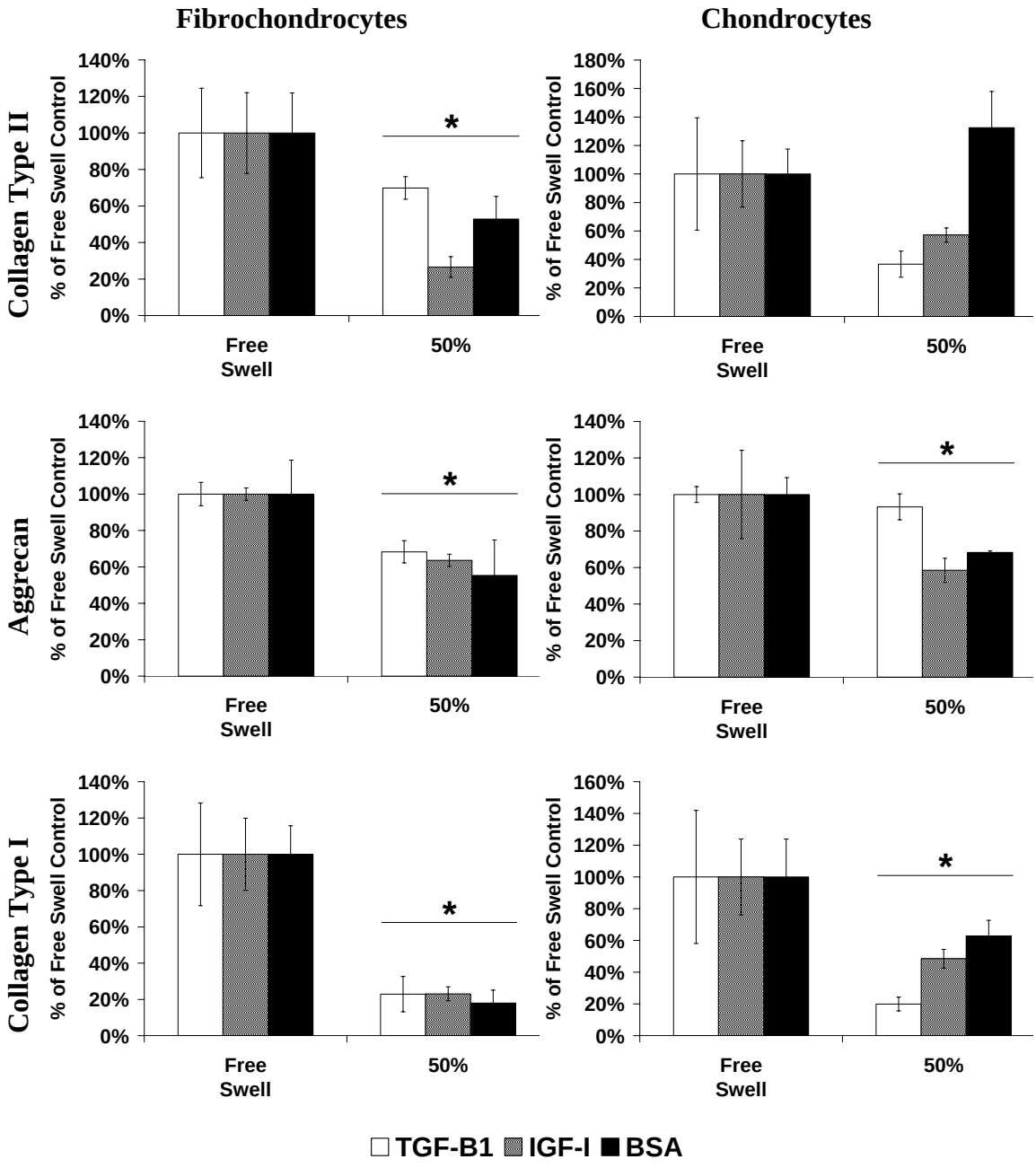

Figure 40: Changes in gene expression of collagen type II, aggrecan, and collagen type I by fibrochondrocytes and chondrocytes seeded in agarose gels. Gels were cultured for 7 days in one of three media conditions: basal/serum-free medium (BSA) or supplemented with either 5 ng/mL of TGF-β1 or 200 ng/mL IGF-I. On the 7th day, gels were placed under 50% static compression. * indicates significant downregulation compared to free swell control expression ($p<0.030$). [n=4 per media condition per compression condition per cell type]

6.3.2 Oscillatory Compression

Whole gels

The effects of intermittent oscillatory compression when applied in concert with cytokine supplementation had similar effects on proline and sulfate incorporation of fibrochondrocytes in agarose gels. The gold standard as previously reported in the literature is the culture environment supplemented with 10% FBS. For this media condition, there was a significant increase in fibrochondrocyte proline incorporation ($p=0.0011$) but not of sulfate incorporation ($p=0.091$) in response to 1.0 Hz intermittent oscillatory compression (Figure 41). Fibrochondrocytes in the basal/serum-free media condition responded as previously described with increases in both proline and sulfate incorporation rates with oscillatory compression ($p=0.0079$ and $p=0.038$, respectively). The results for gels cultured in TGF-β1 were different than expected showing 23% and 40% inhibitions of proline and sulfate incorporation rates ($p=0.0016$ and $p=0.0003$) due to oscillatory compression as compared to 10% static offset. Surprisingly, there were no significant differences between oscillatory loading and the static offset gels in the presence of 200 ng/mL of IGF-I.

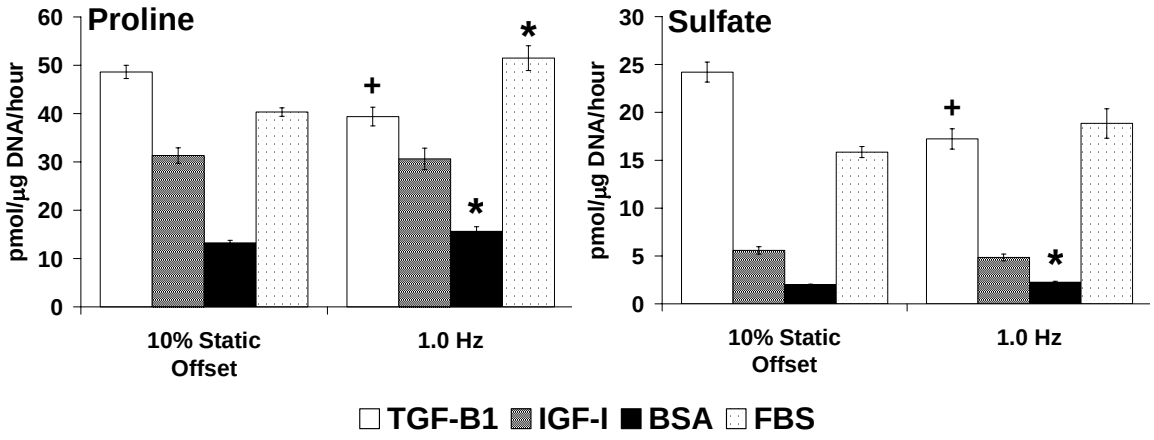

Figure 41: Combined effects of oscillatory compression and cytokine supplementation with either 5 ng/mL of TGF-β1 or 200 ng/mL IGF-I on proline and sulfate incorporation of fibrochondrocytes. Data represents the sum of "ring" and "center" parts of an individual gel, analyzed separately. * indicates significantly greater than 10% static offset ($p<0.038$). + indicates significantly lower than 10% static offset ($p<0.0016$). [n=8 per media condition per compression level]

The chondrocytes in agarose gel culture appeared to respond in a manner similar to what had been previously reported when cultured in 10% FBS (Figure 42). The addition of oscillatory compression stimulated both proline and sulfate incorporation rates over the 10% static control values ($p=0.0005$ and $p=0.0002$, respectively). There were no significant effects of oscillatory compression on the gels that were cultured in the TGF-β1 or basal media conditions. Similar to what was seen for the TGF-β1 media condition for the fibrochondrocytes, the gels cultured in the presence of IGF-I had 11% and 13% inhibitions of proline and sulfate incorporation rates due to oscillatory loading ($p=0.0036$ and $p=0.0009$, respectively).

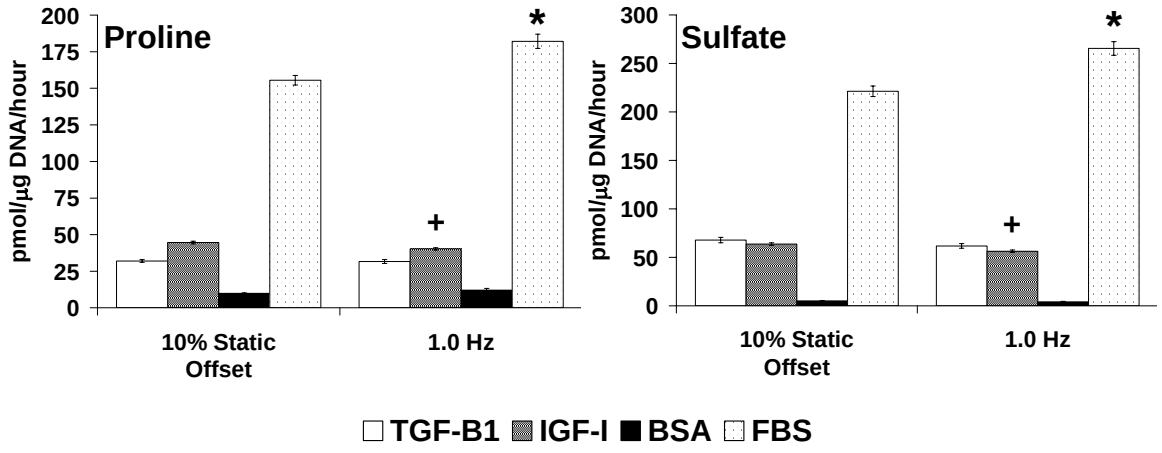

Figure 42: Combined effects of oscillatory compression and cytokine supplementation with either 5 ng/mL of TGF-β1 or 200 ng/mL IGF-I on proline and sulfate incorporation of articular chondrocytes. Data represents the sum of "ring" and "center" parts of an individual gel, analyzed separately. * indicates significantly greater than 10% static offset ($p<0.05$). + indicates significantly lower than 10% static offset ($p<0.05$). [n=8 per media condition per compression level]

Ring vs. center

All samples were separated into "rings" and "centers" and analyzed for biochemical and radiolabel contents separately. In comparing the DNA content of the two regions, the data were normalized by dry mass to account for the difference in volume between the regions (56% ring/44% center). There was not a significant difference between DNA content normalized by dry mass for either cell type under any media condition ($p=0.88$ for fibrochondrocytes and $p=0.39$ for chondrocytes, data not shown), indicating a proportional density of cells between the regions studied. When looking at the matrix distribution, there were striking differences between the media conditions and between the cell types (Figure 43). For all media conditions except for FBS, there was a trend towards greater sGAG production on a per cell basis in the centers

of the gels as compared to the rings. These differences in gels with fibrochondrocytes were significant for the TGF-β1 and BSA media conditions (p=0.019 and p=0.042, respectively). In sharp contrast, the highest amount of sGAG production was in the ring of the fibrochondrocyte gels that were exposed to FBS (p<0.001). The chondrocytes also exhibited a tendency to accumulate greater amounts of sGAG in the center portions of the gels (p<0.001), however, there were no significant differences in the sGAG content between the rings and centers of the FBS group (p=0.83).

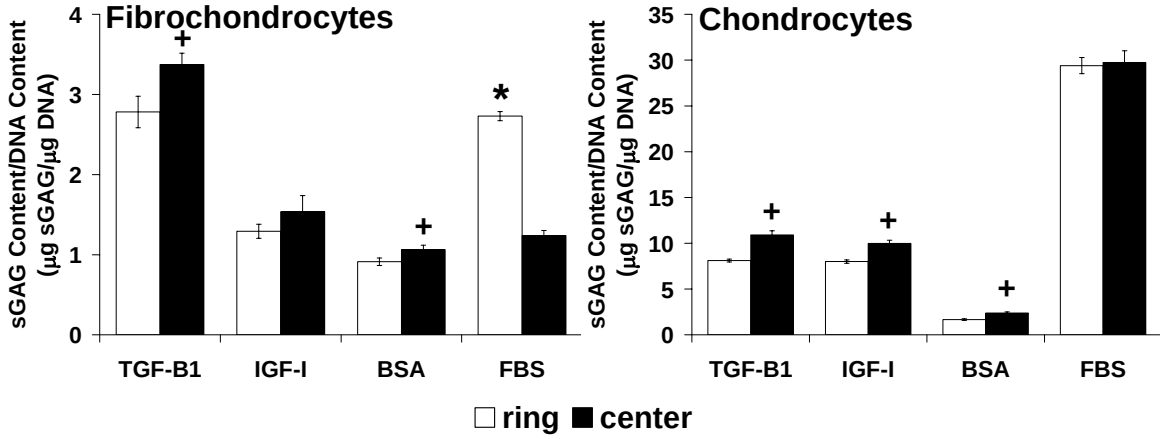

Figure 43: sGAG content on a per cell basis of agarose gels seeded with either fibrochondrocytes or chondrocytes. * indicates significantly greater than center (p<0.05). + indicates significantly greater than ring (p<0.05). [n=24 per media condition per cell type]

The differences in fibrochondrocyte proline and sulfate incorporation rate were examined as normalized to DNA content for ring and center portions of the gels (Figure 44). For each media condition, a two-factor (ring/center and 10% static/1.0 Hz) ANOVA

was performed. Data are plotted as the incorporation rates in the ring normalized by the incorporation rates in the center. There were no significant differences in fibrochondrocyte incorporation rates between rings and centers for gels cultured in either TGF-β1 or IGF-I ($p>0.29$). In comparison, gels cultured in either the BSA or FBS media conditions had significant incorporation differences between rings and centers. The centers of gels cultured in BSA had greater incorporation rates (>18%) independent of loading condition ($p<0.002$). In contrast, the rings of the gels cultured in FBS had greater incorporation rates that were approximately 75% greater than proline incorporation rates of centers and 350%-450% greater than sulfate incorporation rates of the centers ($p<0.0002$).

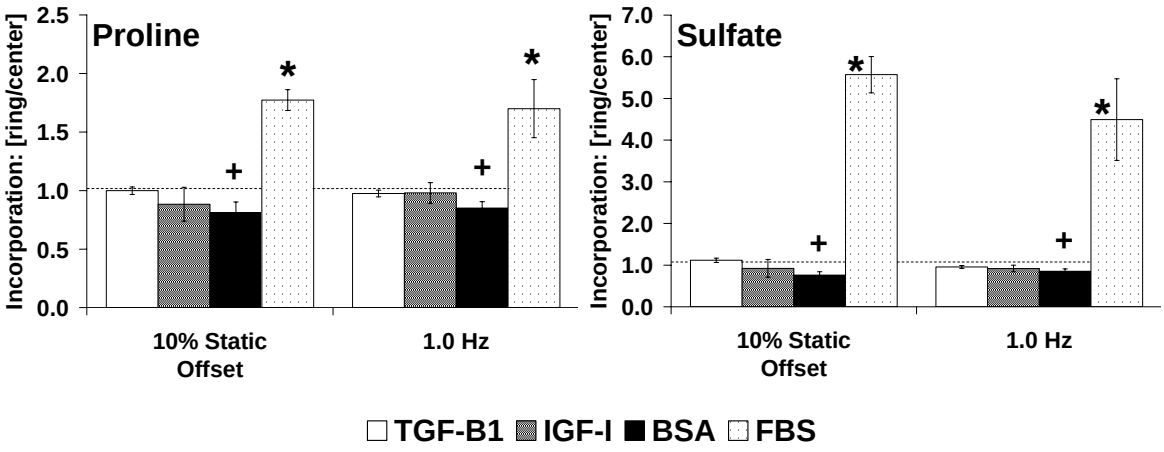

Figure 44: Incorporation rates of fibrochondrocyte proline and sulfate for 6 mm diameter outer rings of the agarose gels normalized by the 4 mm diameter centers. + indicates significantly greater than ring ($p<0.002$).* indicates significantly greater than center ($p<0.0002$). [n=8 per media condition per compression level]

The differences in the distribution of incorporation with the rings seeded with chondrocytes illustrated differences in behavior of the two cell types (Figure 45). The center of gels cultured in IGF-I had significantly greater incorporation rates that were approximately 16%-45% greater than proline incorporation rates of rings and 9%-35% greater than sulfate incorporation rates of the rings ($p<0.0001$). There were different patterns of incorporation rates between proline and sulfate in gels cultured in either TGF-β1 or BSA. Only proline incorporation was significantly different between the ring and center for the TGF-β1 media condition with 20%-33% greater incorporation in the centers ($p<0.0001$). In contrast, only sulfate incorporation was significantly different between the ring and center for the BSA media condition with 8%-35% greater incorporation in the centers ($p<0.0081$). There were no significant differences in either proline or sulfate incorporation rates between the rings and centers of gels cultured in the FBS media condition ($p>0.50$).

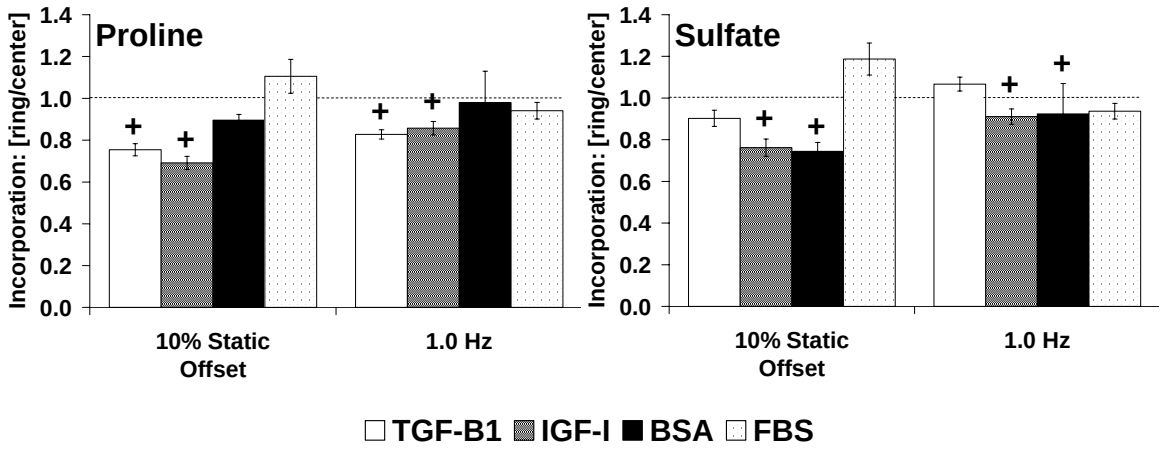

Figure 45: Incorporation rates of chondrocyte proline and sulfate for 6 mm diameter outer rings of the agarose gels normalized by the 4 mm diameter centers. + indicates significantly greater than ring ($p<0.0081$). [n=8 per media condition per compression level]

6.4 DISCUSSION

In addition to biochemical factors, biomechanical stimuli aid in directing the development and maintenance of the tissues within the knee joint. Bonassar *et al.* examined the combined effects of mechanical compression and IGF-I treatment on immature bovine articular cartilage explants. Their first set of studies looked at the addition of 300 ng/mL of IGF-I to cartilage explants at 0% and 50% compression. IGF-I stimulation of both protein and proteoglycan synthesis was reduced with the addition of 50% static compression[14]. Overall, the kinetics of action of either inhibition by static compression or stimulation by IGF-I did not change when used in combination. A second set of studies showed that the addition of oscillatory compression substantially

reduced the time to steady-state stimulation by IGF-I, but this response was still substantially slower than the biosynthetic response to oscillatory compression alone[15]. Taken together, both studies support the idea that the stimulation of articular cartilage by IGF-I and mechanical compression occur through separate cellular mechanisms, with the primary interaction being inhibition (static) or enhancement (oscillatory) of IGF-I transport through the matrix.

Our results with meniscus tissue explants are consistent with this hypothesis. For all four growth factors we observed an inhibition of matrix accumulation relative to free swell controls with the addition of static compression at levels of 0%, 25%, and 50%. Despite the wide range in stimulatory potentials, the relative inhibition by static compression was comparable for all four growth factors. This inhibition was level-dependent for proline incorporation and level independent for sulfate incorporation. This may indicate that, regardless of biochemical stimulation, the inhibition caused by the biomechanical stimulus has a greater and more potent effect on the matrix production of the cells. Our results appear to be consistent with the notion that fibrochondrocytes in their native matrix respond to biochemical and biomechanical stimuli via separate cellular pathways. Short term kinetic studies examining the initial kinetics of stimulation with and without mechanical compression would further clarify this issue.

Several studies have been performed to further address the specific intracellular pathways of action of chondrocytes in response to mechanical load and growth factor supplementation. Fanning *et al.* have looked at the activation of specific MAPK pathways in response to static compression or IGF-I on articular cartilage explants[150]. Their studies showed different profiles (temporal and in magnitude) of phosphorylation

of the ERK1/2, p38, and JNK(SEK1) pathways in response to 50% static compression. This suggests the importance of these pathways in mechanotransduction, as well as, individual activation of each as seen in the differences in temporal activation. The activation of ERK1/2 in response to IGF-I occurred within 10 minutes and was transient in nature, unlike the sustained phosphorylation (through 24 hours) of ERK2 seen in response to static loading. These results indicate the existence of distinct mechanotransduction pathways in response to biochemical or biomechanical stimuli.

As discussed in Chapter 4, there are several proposed mechanisms by which the fibrochondrocytes sense static compression. In revisiting these mechanisms, we can apply them in the context of modulating growth factor supplementation in both matrix environments. One proposed mechanism that would modify cell response to static compression is altered transport of biochemical factors and waste factors due to matrix compaction. Decreased solute diffusivity in articular cartilage explants due to static compression has been studied in detail for the transport of dextrans. Quinn *et al.* measured significant decreases in dextran diffusivity and partitioning due to 23% static compression of articular cartilage explants[151]. Dextrans provide a model for the fluorescent tracking of molecules and are on the same order of magnitude in size as the growth factors used in the current studies. These results indicated a change in the kinetics and concentrations of growth factor delivery under static compression.

In the agarose gels, it appeared that any diffusion effects on transport due to compression did not significantly affect the biosynthesis as the rates of the “rings” compared to those of the “centers” were comparable for all gels with an exception of the FBS treated fibrochondrocyte agarose gels. However, changes in partitioning could be

partially responsible for matrix synthesis inhibition. In addition to the dextran data, Bonassar *et al.* have shown a significant decrease in the equilibrium concentration of ^{125}I-labeled IGF-I in cartilage explants held at 50% compression[14]. This overall decrease in growth factor concentration could lower matrix synthesis rates relative to unstrained controls. Our findings appear to suggest that in the current system, the availability of the growth factors is a limiting factor in matrix synthesis inhibition. Recalling the data from the previous chapter, the concentrations of TGF-β1 studied did not reach a plateau in fibrochondrocyte matrix synthesis within the range of concentrations chosen, therefore 5 ng/mL was chosen as a feasible concentration for the subsequent studies. In contrast, 200 ng/mL of IGF-I is within the plateau range of matrix synthesis, where for all concentrations, a similar response in matrix synthesis occurs. With the addition of static compression, amounts of matrix synthesis inhibition are greater for TGF-β1 than for IGF-I relative to respective free swelling controls for either fibrochondrocyte culture environment (explants or gels). This suggests that changes in growth factor concentration could contribute to the modulation of matrix synthesis under static compression.

The response of chondrocytes in agarose gels to static compression was not consistent with these findings. It is difficult to draw firm conclusions based on the chondrocyte data due to the reduced sample size. Additionally, since we did not perform dose-response studies on the agarose gels, it is unclear where on the matrix synthesis curve these concentrations lie. Based on articular cartilage explant literature, it is likely that both concentrations are within the increasing portion of the matrix synthesis curve, not present in excessive amounts[11,79].

Another cellular mechanism of sensing static compression is the increase in fixed charge density due to static compression. This in turn causes a decrease in pH which has been shown to inhibit chondrocyte matrix synthesis[119]. Additionally, changes in pH have been shown to change growth factor activities. Changes in pH towards an acidic environments have been shown to decrease IGF-I binding affinity for its receptor on chondrocytes[152]. In contrast, acidic environments can also activate latent TGF-β1 into its active form. In the current studies, this proposed change may not be applicable to our culture system. As the fixed charged density as related to the abundance of proteoglycans, it is much lower in both the meniscus tissue explants and the early time-point agarose gels and would not be increasing significantly to induce the inhibition of matrix synthesis.

A final proposed mechanism by which cells sense static compression is changes in cell shape as dictated by cell-matrix interactions. Lee and Bader have shown increases in cellular deformation in agarose gels that are inversely related to amount of matrix present[153]. Our agarose gels have a low amount of total accumulated matrix allowing for significant amounts of deformation, but also inhibiting the cells from immediately returning to their original shape during relaxation. In contrast, matrix synthesis was measured over a 21 hour period, whereas cellular deformation and return to an unloaded shape would have occurred on the order of several hours, suggesting that the dominating effect of matrix synthesis inhibition was not cell deformation in the agarose gels. However, in the meniscus explant model, a direct link between fibrochondrocytes and matrix components is present. With the application of static compression, decreases in chondrocyte volume and surface area have been correlated to decreases in sulfate

incorporation rates[117,118]. As the cell projections of the fibrochondrocytes have been shown to be fully integrated into the extracellular matrix[34], it is likely that static compression of the explants induces sustained changes in cell morphology that could be responsible for decreases in matrix synthesis. Additionally, the growth factor actions on cells that are responding to physical strain may be different to what is seen in the unstrained case through the expression of specific integrins on the cell surface. Mechanical load has been shown to increase the number of integrins, specifically $\alpha 5$ and $\beta 1$ subunits, in response to oscillatory stress[154,155]. Chowdhury *et al.* has shown that the stimulation of human chondrocytes under oscillatory compression is dependent on an integrin ($\alpha 5\beta 1$) mediated TGF-$\beta 3$ response. This is another component of the possible cell-matrix interactions, however, the analyses of current studies do not lend themselves to drawing conclusions about the role of integrins in our model.

Changes in gene expression due to 50% static compression compared to free swell controls were also examined. The 0% compression group would be a more appropriate control with the same diffusional limitations; however, the free swell group was chosen to account for all effects of static compression. In the methods section, it was noted that the gels used for this study were seeded at an increased cell density of 20×10^6 cells/mL. This higher cell density was used so that gels did not need to be pooled to obtain a single sample for RNA isolation and subsequent PCR analysis. Gels at the normal cell density of 5×10^6 cells/mL were cultured in BSA and run in parallel to provide a comparison of baseline gene expression between the two cell densities. With the exception of one group, for each cell type there were no significant differences in baseline expression between the high density and low density gels ($p>0.18$) for the three genes studied. The

high cell density gels seeded with chondrocytes had increased levels of collagen type II expression. This could be due to differences in cell-cell or cell-matrix interactions. Increases in these interactions may occur in due to the increased number of cells present.

Overall, there was significant downregulation of each gene for both cell types due to 50% static compression ($p<0.030$) with one exception. There was not a significant effect of static loading on collagen type II gene expression of chondrocyte seeded agarose gels. Upton *et al.* have shown downregulation of collagen types I and II gene expression of adult porcine fibrochondrocytes in tissue explants in response to static loading. In contrast to our current findings, they showed no effect of static loading on aggrecan expression, but found significant downregulation of decorin. These differences could indicate that the fibrochondrocytes in the current study when exposed to growth factors in agarose gels may be responding in a more "chondrocytic" manner with regulation of aggrecan. From a morphological point of view, the fibrochondrocytes are not actively attached to their agarose matrix, remaining in a rounded shape, similar to what is seen in the inner one-third of the meniscus[34]. This could also contribute to the "chondrocytic" behavior of the cells. Determining changes in biglycan and decorin gene expression may aid in understanding the "fibrochondrocytic" response in the 3-D gel.

The gene expression results are subject to normalization with a housekeeping gene such as GAPDH, 18S rRNA, or β-actin, which would be a more acceptable normalization technique. Housekeeping genes serve as internal controls to account for potential fluctuations in RNA due to different treatments. Although the expression levels of these genes have been shown to change during differentiation of keratinocytes[156] and stem cells[157], levels within a single treatment group have been consistent. Using the

$\Delta\Delta C_t$ method, as suggested by the manufacturer of the thermocycler, the C_t value or cycle number of the housekeeping gene is subtracted from the C_t value of the target gene for each sample, accounting for changes in RNA content of the sample. These ΔC_t values of the treatment can then be compared to the ΔC_t values of the controls, providing a normalized method of analysis of real-time quantitative RT-PCR. Future work includes developing a set of bovine primers for one of these housekeeping genes.

Lastly, the effect of an intermittent oscillatory compressive protocol was studied for the agarose gels after a week in culture with either TGF-β1 or IGF-I. Buschmann *et al.* have shown a stimulatory response of early (day 2) agarose gels seeded with chondrocytes under 1.0 Hz compression[73]. For the media condition that is the closest to what was used in their study, we saw a significant increase in matrix synthesis with the application of 1.0 Hz intermittent oscillatory compression in the chondrocyte seeded agarose gels grown in the FBS media condition, corroborating well with their results. However, this behavior was not conserved across the other media conditions or cell type studied. For fibrochondrocytes in the presence of FBS, only protein synthesis was stimulated in response to oscillatory compression, similar to what was seen for fibrochondrocytes in tissue explants under a modified oscillatory compression protocol. Interestingly, there was an inhibition of matrix synthesis with the addition of oscillatory compression for the TGF-β1 fibrochondrocyte seeded gels as well as the IGF-I chondrocyte seeded gels compared to their respective 10% static compression controls. The variation in the effects of oscillatory compression may be dependent upon amount of accumulated matrix, which is directed by the media formulation. From the time-course data characterizing agarose gel maturation (Chapter 5), the chondrocyte seeded agarose

gels had the greatest matrix accumulation by day 7 and the highest matrix synthesis rates at day 7. This chondrocyte behavior in agarose is similar to what has been shown previously in agarose gels[73] and tissue explants[69,71,72], suggesting a minimal requirement for maturation in order for chondrocytes to respond to oscillatory compression. This would implicate the importance of cell-matrix interactions as a major mechanism for increased synthesis in response to oscillatory loading.

In contrast, the fibrochondrocyte seeded gels showed stimulation of matrix synthesis with oscillatory compression for the basal/serum-free media condition. This finding contrasts the lack of chondrocyte response to oscillatory compression in the basal media condition. However, this difference could indicate a difference in mechanotransduction mechanisms between the fibrochondrocytes and the chondrocytes, suggesting an increased sensitivity of fibrochondrocytes over chondrocytes to mechanical load in this basal environment. One reason for this could be the change in shape induced by agarose culture. Fibrochondrocytes in agarose morphologically resemble the shape seen in the compressive inner one-third of the meniscus. As shown in Chapter 3, meniscus explants from the inner regions of either meniscus had significantly greater expression of collagen type II and aggrecan as well as increased proline and sulfate incorporation rates over explants from the outer region. Taken together, this finding may suggest of a relationship between fibrochondrocyte shape and increased propensity towards matrix deposition.

These studies also contain a comparison between the outer “rings” and inner “centers” of the agarose gels. In these studies, all samples were separated into ring and center and analyzed separately. There was an interesting distribution of sGAG deposition

across the diameter of the gels that varied with media condition and cell type. For the well characterized system of chondrocytes seeded in agarose cultured in 10% FBS, there was no difference in the content of sGAG on a per cell basis between the ring and the center. However, for all other media conditions, there was a significantly greater sGAG content in the center portion of the gel compared the outer ring. For the fibrochondrocyte seeded agarose gels cultured in FBS, as seen in the static compression studies, there was a significantly greater sGAG content in the rings of the gels compared to the centers. For the other media condition, fibrochondrocytes behaved similar to chondrocytes, depositing greater amounts of sGAG in the centers of the gels.

One explanation for this difference could be changes in cell viability. The Hoechst dye assay performed to quantify cell number relies upon the binding of the Hoechst to double-stranded DNA, not capable of discerning between DNA from live or dead cells. Live-dead staining was not done on these gels and will be done in the future to clarify the effects of media formulation on cell viability. An alternative reason for preferential sGAG deposition in the center of the gels could be the presence of a secondary cell population. As the digest protocols of both the fibrochondrocytes and the chondrocytes yields a heterogeneous population of cells, the cells on the surfaces of the gels may take on a more "fibroblastic" phenotype, as seen during the dedifferentiation in 2-D monolayer culture[85]. This has been shown in chondrocyte seeded agarose gels with the development of a thin layer of cells on the gel surface. With increased culture duration, this layer became thicker and more dense[87]. Additionally, Mauck *et al.* saw a change in gross appearance of the surface of their chondrocyte seeded agarose gels in the presence of TGF-β1. Under light microscopy, the development of cell masses was

clearly responsible for the surface irregularities[16]. As the outer ring portion contains a greater fraction of the gel's external surface area, the effects of the potentially fibroblastic cells may influence the measured responses from that portion of the gel. Future work includes histological analysis of the agarose gels to map the distribution of the matrix (safranin O for proteoglycan and Masson's Trichrome for collagen) and cells (eosin).

Due to the open questions about the distribution of viable cells and matrix deposition, it is difficult to draw firm conclusions about potential transport mechanisms based on the ring and center data. Increases in matrix synthesis in the outer ring as compared to the inner center have been identified for chondrocyte seeded agarose gels[73] and cartilage tissue explants[72,117] under oscillatory compression. Mechanisms identified as responsible for these preferential biosynthesis increases include increased fluid flow at the surface of the gels that would increase transport of larger solutes and provide a secondary fluid shear stimulation of the cells. Although the current studies agreed well with what has been previously reported for whole agarose gels seeded with chondrocytes and cultured in FBS, we did not see enhanced rates of matrix synthesis in the outer rings of the gels, as would be expected if fluid flow or enhanced convective transport were responsible for stimulating the cells. In contrast, there was a significant enhancement of matrix synthesis in the ring of fibrochondrocyte gels in FBS that is likely also not due to increased convective transport of large solutes due to comparable increase in the rings of the gels held at 10% static offset. The heterogeneity in matrix synthesis of the fibrochondrocytes in agarose gels appears to be independent of loading, as even free swell groups in both static and oscillatory loading studies had greater incorporation rates of both proline and sulfate in the ring.

In contrast to the behavior of gels cultured in FBS and subjected to oscillatory compression, there were two media conditions for which there was an inhibition of matrix synthesis with the addition of oscillatory compression: fibrochondrocytes in TGF-β1 and chondrocytes in IGF-I. This finding was unexpected knowing the characterized effects of oscillatory compression on chondrocytes. A possible hypothesis for this could be a switch in the effects of the loading protocol from matrix synthesis to cellular proliferation. Chowdhury *et al.* found increases in cellular proliferation in response to short duration (1.5 hours) loading times. Although we did not find a significant increase in DNA content of the gels, it believed that chondrocytes pushed into the S-phase of cell cycle may take several days for a that cycle to complete. Future studies could explore the differences in ^{3}H-tymidine uptake.

In conclusion, these studies examined the combined effects of mechanical loading and growth factor supplementation on fibrochondrocytes in tissue explants and agarose gels. Static compression up to 50% had greater inhibitory effects on fibrochondrocytes in tissue explants with all levels of compression inhibiting matrix synthesis relative to free-swelling controls. Fibrochondrocytes in agarose gels were only inhibited with the application of 50% compression. However, there were similar trends in the relative levels of inhibition for a specific media treatment between matrix environments with greatest changes in inhibition with TGF-β1 relative to free swell controls. Additionally, chondrocytes seeded in agarose gels behaved in a similar manner to the fibrochondrocytes in agarose, with matrix inhibition only occurring at 50% static compression. In comparing the growth factor treatments, it appeared that inhibition of matrix synthesis by static compression was the greatest relative to free swell controls for

IGF-I. These results are suggestive of separate cellular pathways of action in response to each stimulus. The effects of oscillatory compression coupled with growth factor supplementation were also studied for fibrochondrocytes and chondrocytes in agarose gels. In response to free swelling culture alone, there was a heterogeneous deposition of matrix components that varied with media treatment and cell type. The addition of oscillatory compression did not enhance matrix synthesis in all conditions. These findings show differences in the behavior of fibrochondrocytes in their native matrix and hydrogel scaffold. There were also differences within preculture conditions, suggesting that choice of culture conditions can direct the development of fibrochondrocyte matrix deposition in the agarose hydrogels.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

The menisci have been identified to play a major role in the maintenance of a healthy knee joint, providing load transmission, joint stability, and lubrication. Despite the organs' importance in maintaining normal knee biomechanics, there are few reports characterizing the behavior of meniscal fibrochondrocytes in response to physiologically relevant stimuli. Much of the literature involving 3-D scaffolds for meniscal regeneration and the use of different fixation techniques to induce reparative responses uses difficult to control *in vivo* models. As the current literature also implicates degeneration of the menisci during the early pathogenesis of osteoarthritis, the menisci may also become a target for therapeutic intervention. In order to implement any repair or regenerative strategies, it is important to understand the normal baseline behavior of the cells in their native environment to be able to obtain criteria for efficacy. To this end, the work in this thesis examined the effects of biomechanical and biochemical stimuli, individually and in combination.

The studies presented in Chapter 3 provided a general characterization of the immature bovine models used in the thesis. Similar to reports for menisci from other species, the extracellular matrix composition of the immature bovine meniscus was found to be heterogeneous across the radial cross-section of the tissue. Additionally, there were differences in fibrochondrocyte gene expression between the outer regions and inner regions with significantly greater expression of collagen type II in the inner regions and

decorin in the outer regions. Despite these inherent differences, when placed in a fibrin scaffold fibrochondrocytes from different regions appeared to converge to a similar endpoint after two weeks in culture. Finally, there were very consistent trends among tissue explants from the inner and outer regions of both menisci in response to the stimuli studied in this thesis. These findings characterized the model used in this thesis. They also provided justification for the use of middle-outer zone tissue for explants as overall there were no differences in the general trends in responses of regional explants to the external stimuli and no differences in responses of explants from the outer regions. Additionally, they provided justification for the use of pooled cells from all regions, as the cells placed in a 3-D scaffold appeared to behave similarly.

The studies presented in Chapter 4 explored the biosynthetic effects on meniscal fibrochondrocytes of specific biomechanical stimuli. The effects of physiologically relevant static and oscillatory compression were studied using custom designed mechanical loading devices. Meniscus and cartilage tissue explants placed under static compression up to 50% of original cut thickness responded similarly with comparable levels of inhibition of protein synthesis due to 25% or 50% compression, suggesting that fibrochondrocytes and chondrocytes sense and respond to static compression via similar mechanisms. Static compression did not modulate matrix biosynthesis of fibrochondrocytes or chondrocytes seeded in agarose gels; however this response may be dependent on the culture media formulation. Oscillatory compression again modulated protein synthesis similarly between meniscus and cartilage explants, supporting a common relationship in sensing mechanical load and protein production between the cell types. When seeded in agarose gels, chondrocytes were insensitive to oscillatory

compression, while fibrochondrocytes had increased matrix synthesis. These findings illustrated differences in cell behaviors that appear to be dependent at least partially on their matrix environment. Additionally, in either matrix environment, fibrochondrocyte proteoglycan synthesis rates were at least an order of magnitude lower than those of chondrocytes.

The studies presented in Chapter 5 explored the biosynthetic effects on meniscal fibrochondrocytes of specific biochemical stimuli in the absence of any mechanical loading. The studies focused on anabolic cytokines or growth factors that typically enhance matrix production, specifically TGF-β1, IGF-I, PDGF-AB, and bFGF. Matrix synthesis in meniscus tissue explants was stimulated by all factors. In particular TGF-β1 was the most potent factor (in explants and gels) and produced a greater fold stimulation of matrix synthesis than has been reported for similarly aged articular cartilage. Trends in stimulation of fibrochondrocytes in either matrix environment were generally similar. However, for the concentrations of cytokines used, neither cytokine stimulated chondrocyte matrix synthesis above the level achieved in 10% FBS supplemented medium. These studies illustrated the ability of fibrochondrocytes to produce *de novo* matrix with content that is dependent upon biochemical supplementation. Additionally the same stimuli affected the chondrocytes in a distinct manner highlighting the innate differences between these two cell types in native matrices or 3-D scaffolds. Specifically, the TGF-β1 supplemented medium was the most potent stimulator of fibrochondrocyte matrix synthesis, whereas the 10% FBS supplemented medium was the most potent for chondrocytes.

Finally, Chapter 6 addressed the effects of the combination of these stimuli on meniscus tissue explants and fibrochondrocytes and chondrocytes in agarose gel culture. For these studies, it was hypothesized that there would be a synergistic action between the superposition of biomechanical and biochemical stimuli. For synergy to occur, a combined action of the two combined stimuli would cause an effect on matrix biosynthesis that was greater than the additive effect of either stimulus applied individually. Increases in static compression levels led to level-dependent inhibition of protein synthesis and level independent inhibition in proteoglycan synthesis in meniscus explants. Additionally, there was an inhibition of proteoglycan synthesis that, when scaled by the free swelling synthesis rates for each media formulation, was not significantly different among culture media conditions. This is consistent with the proposal of interacting pathways by which cells respond to either biomechanical or biochemical stimuli. Matrix synthesis of agarose gels seeded with either cell type supplemented with growth factor and subsequently statically compressed was only inhibited at 50% static compression. As these gels had developed a very immature *de novo* matrix after 7 days of preculture, a likely contributor to matrix synthesis inhibition was the reduction in solute diffusivities due to 50% compression or direct cellular deformation. In contrast, the addition of oscillatory compression significantly stimulated matrix synthesis of chondrocytes seeded in agarose and cultured in 10% FBS, agreeing well with the literature. Fibrochondrocytes in 10% FBS responded with increases in protein synthesis, consistent with meniscus explant data. Surprisingly, oscillatory compression inhibited matrix synthesis for chondrocytes in IGF-I supplemented media

and for fibrochondrocytes in TGF-β1 supplemented media, but further studies would be required to determine the cause of these inconsistent responses.

From the results of these studies, several inferences can be made about differences between fibrochondrocytes and chondrocytes. In an explant culture system, there was an overall difference in baseline matrix synthesis rates between the two cell types. Total protein synthesis rates were comparable. In contrast, the proteoglycan synthesis rates of chondrocytes were an order of magnitude greater than those of fibrochondrocytes. This finding was consistent with the order of magnitude difference in overall proteoglycan content of the two tissues. In response to mechanical loading, there were no effects on the proteoglycan synthesis rates of fibrochondrocytes, suggesting that enhanced proteoglycan turnover in the tissue may not occur in response to mechanical loading. As the mechanical loading protocols in these *in vitro* studies are a simplified model of *in vivo* loading, there may be other mechanical components of *in vivo* loading that may direct proteoglycan turnover that are not significant components of the current protocol. One important component may be hydrostatic pressure. Due to differences in geometry and boundary conditions, pressure levels generated during physiological loading of native tissue are three orders of magnitude greater than what is seen in these *in vitro* experiments. To obtain more physiologically relevant levels of pressure, direct application of hydrostatic pressure can be used. Cyclic hydrostatic pressure has been shown to increase proteoglycan synthesis of articular chondrocytes in explant culture[64,158]. Additionally, these studies isolate the effects of increased pressurization alone without inducing fluid flow or tissue deformation in the sample.

For both cell types seeded in agarose gels and exposed to various media conditions, hyper-physiological growth factor supplementation appeared to cause a more potent stimulation of matrix synthesis compared to what was shown for mechanical compression. This finding suggests an increased importance of biochemical stimuli in directing the development of matrix synthesis as compared to biomechanical stimuli that is potentially important in optimizing tissue engineering protocols. However, as seen in the comparison of baseline synthesis between cell types in tissue explants, the maximal levels of sGAG accumulation and proteoglycan synthesis rates of the fibrochondrocytes only were able to reach the same order of magnitude of the accumulation and synthesis of the chondrocytes cultured in the basal/serum-free media condition. Growth in media supplemented with TGF-β1 showed the greatest effects on increasing fibrochondrocyte matrix synthesis rates and matrix accumulation, with no plateau in biosynthetic stimulation of meniscus explants with up to 100 ng/mL of TGF-β1. As these cells have been identified to illustrate both fibroblastic and chondrocytic behaviors, their propensity towards one phenotype over another is currently unknown. At 100 ng/mL of TGF-β1, there appeared to be increasing levels of both protein and proteoglycan synthesis of fibrochondrocytes in explant culture. Additionally, after 14 days of exposure to 5 ng/mL of TGF-β1, the fibrochondrocytes in agarose gels appeared to upregulate expression of chondrocytic genes. This observation is suggestive of TGF-β1 directing the behavior of fibrochondrocytes towards that of chondrocytes. Future studies would be needed to test the reversibility of the effects of TGF-β1 on this behavior as an indication of differentiation, as well as determining an upper limit in biosynthetic response of the fibrochondrocytes to increasing concentrations of TGF-β1.

In the two different matrix systems studied, fibrochondrocytes had similar responses to oscillatory compression. In explants or agarose gels cultured in media supplemented with 10% FBS, there were significant increases in protein synthesis, but not proteoglycan synthesis with oscillatory compression. This finding in both culture systems may suggest that the mechanical stimulation imposed in these studies is not a dominant factor in regulation of proteoglycan synthesis. In contrast to similar biosynthetic responses to compression, the response of fibrochondrocytes in the two matrix systems differed with growth factor supplementation. Although both the newly excised explant disks and agarose seeded gels were precultured for 3 days in basal/serum-free medium to allow for equilibration, there were two distinct time-course profiles in biosynthesis with the addition of growth factors. Protein synthesis was stimulated over synthesis of BSA controls with the addition of growth factors in the agarose gel system but not in the tissue explants over the 2 week culture duration. Additionally, the temporal changes in proteoglycan synthesis rates were different between culture systems. Synthesis rates of fibrochondrocytes in explants were stimulated above BSA controls at day 2 and remained fairly consistent among all time points examined. This was in contrast to the temporal profile of the agarose gels showing a steep increase in matrix synthesis through the first four days of culture followed by a leveling off of synthesis rates through the remaining time in culture. The agarose gel is more permeable than the tissue; therefore limitations in diffusion would not be expected. The low initial synthesis rates followed by a sharp increase in synthesis may indicate an equilibration of the fibrochondrocytes to this matrix environment. It is unknown how the cells respond to the collagenase digestion process and subsequent seeding into a 3-D scaffold, but it may

involve a change in cell receptor and integrin profiles, which would affect reactivity of the cells to external stimuli such as growth factors. These cellular changes could also be responsible for the biosynthetic differences seen in response to growth factors between fibrochondrocytes in tissue explants and in agarose gels. Immunohistochemical staining for cell surface growth factor receptor profiles in both tissue and gels could help explain the delayed stimulation of matrix synthesis to continued exposure to growth factors for fibrochondrocytes in agarose gels.

It is also important to recognize the difference between macroscopic effects and local effects of these external stimuli. The overall macroscopic strain imposed on a sample is translated into a local strain based upon sample composition that will directly affect the cell. The sample composition differs highly between the explant and agarose gel culture systems. Based upon the origin of isolation, the fibrochondrocytes in tissue explants have a stellate morphology with interactions with the developed extracellular matrix. When placed in agarose gels for the time points studied, the morphology is likely more like that of rounded chondrocytes in the middle zone of articular cartilage, surrounded by a pericellular matrix that does not have much interterritorial interaction. In response to compression, a stress shielding event may occur, where the bulk of the compression is absorbed by the agarose matrix, making the local strain seen by the cells very different from the macroscopic applied strain. A different strain magnitude may also be seen by the cells in the native matrix as they may have increased interactions with their surrounding matrix components. To explore the importance of cell-matrix interactions in regulating biosynthetic responses to mechanical stimuli, future studies could measure biosynthesis of agarose gels of different culture durations as matrix

deposition extends into the interterritorial space between cells with time in culture. Additionally, studies exploring different 3-D scaffolds to promote different cell-matrix interactions (e.g., fibrin gels or polyglycolic acid scaffolds) could be used to examine contributions of cell-matrix interactions on the response to compression.

Similar to translation of macroscopic strains to local strains, an inherent difference in certain matrix components between the two culture systems could produce a local growth factor concentration that is different from the supplemented growth factor concentration. The tissue matrix potentially has an increased density of matrix binding proteins that can interact with the available growth factors, sequestering them from the cell receptors. Therefore, the actual concentration of available growth factor may be lower than the concentration in the feed media. Again, as the matrix content is lower in the developing agarose gel, the presence of such binding factors is likely to be less than what is found in the tissue matrix. As previously mentioned in the discussion of the differences in the time-course responses of fibrochondrocytes in tissue explants and agarose gels, the cell surface receptor profile may differ between the two culture systems. Immunohistochemical staining can be used to probe for binding proteins that interact with matrix components and for cell surface receptor profiles. Additionally, an ELISA can be developed to quantify binding proteins that may also exist in the free culture medium, not bound to a matrix component.

As previously discussed, there are multiple mechanisms by which the cells may sense mechanical stimulation. The application of the current oscillatory loading protocol induces a complex physical environment that includes spatial distributions of biophysical phenomena, such as fluid flow or hydrostatic pressure, that have been shown to correlate

with cellular biosynthesis within the tissue explants or gels due to oscillatory compression[72,73,78]. Cell-matrix interactions and physical cellular deformation have also been identified as potential factors that may influence matrix production of cells in response to mechanical load. To address specific contributions of cell-matrix interactions, shear deformation can be applied[66,143]. In this type of stimulation, the sample experiences minimal volumetric changes with very small variations in fluid flow or fluid pressurization. Additionally, convective transport of growth factors has been shown to be negligibly affected in this model. This type of external stimulus could provide an alternative method for studying the differences in biosynthesis of rings and centers of the agarose gels. With the omission of a distribution in fluid flow, it is likely that the increase in matrix synthesis seen in the 10% FBS medium condition for the gel centers would not be present, suggesting the importance of fluid flow in the delivery of serum macromolecules.

These studies utilized a single aged animal model, however changes in meniscal matrix content and fibrochondrocyte behavior that have been identified to occur with aging[97,98,159,160] may influence the response of aged tissue when exposed to the same stimuli of the current studies. It is likely that with an increase in age, as seen in studies comparing immature and mature articular cartilage explants, responsiveness to specific biomechanical[104,161] or biochemical[82,162] stimuli will decrease. Characterizing the extent to which the cells become desensitized to stimuli with age may indicate a permanent loss in biosynthetic activities. In the case of the fibrochondrocytes, aging may shift the cells towards a terminal phenotype that is more fibroblastic, in contrast to the results in the current studies suggesting a shift towards a chondrocytic phenotype with exposure to

TGF-β1. Additionally, the response of the cells to the external stimuli may be related to the changes in the matrix composition, which in turn would affect the matrix-cell interactions that aid in sensing mechanical stimuli. Parallel studies to the current studies using tissue from older animals will provide an understanding of changes in sensitivity to similar physiologically relevant stimuli. Using adult tissue may provide a more relevant model for understanding response of a tissue that may be subject to degenerative changes

These studies have shown the biosynthetic responses of fibrochondrocytes in native tissue explants to physically relevant mechanical stimuli. The extent to which these cells respond to these different stimuli provides a basis for understanding the normal homeostatic activity of the cells. In the healthy *in vitro* environment studied, there were no effects of mechanical stimulation on proteoglycan synthesis, suggesting the importance of other types of stimuli such as very specific mechanical cues or other biochemical cues for the production of proteoglycans. Additionally, differences in fibrochondrocyte biosynthesis were observed between the two culture systems studied, indicating a varying potential of fibrochondrocyte response to external stimuli that may be matrix dependent. This result was specifically seen in the greater response in stimulation of the fibrochondrocytes in agarose gels over that seen in native tissue when subject to biochemical supplementation. These findings are important to understanding *in vitro* development of 3-D scaffolds for meniscal regeneration, recognizing that the cellular behavior may be highly dependent on scaffold choice and may differ from that of behavior in the native environment.

Taken together, these data illustrate differences in the behaviors of fibrochondrocytes in distinct matrix environments to exogenous stimuli. As noted, there

is currently one published study looking at the behavior of fibrochondrocytes in agarose gel culture. This inert 3-D scaffold supports a rounded cell morphology, and in the presence of the required media environment, may direct fibrochondrocyte towards a chondrocytic behavior as seen in the preliminary real-time quantitative RT-PCR data. Additionally, these studies provide a characterization of the immature bovine meniscus, first from a general approach, providing compositional information and matrix distribution images useful for other groups using the same animal model. Overall, these *in vitro* studies have provided valuable information about the mechanobiology of fibrochondrocytes within two distinct culture systems when exposed to biomechanical and biochemical components of physiological stimuli. Parallelisms drawn between the behavior of fibrochondrocytes and chondrocytes begin to offer a more global consideration of the knee joint, showing that there are distinct behaviors of the two cell types to identical stimuli. These distinct behaviors may be dependent upon a difference in inherent cellular behaviors, differences in local matrix interactions in their native environments that dictate perception of a stimulus, or most likely a combination of both of these things.

7.2 Recommendations and Future Work

The work presented in this thesis has added to the current body of knowledge on the meniscus and brings forth many interesting points of discussion. Coupled with the extensive literature on articular cartilage, these studies begin to create a foundation for understanding and comparing two important load bearing structures in the knee joint.

These studies provide a characterization of fibrochondrocytes in native explants and in 3-D scaffolds in response to specific biomechanical and biochemical stimuli. Fibrochondrocytes were once thought to be quiescent and incapable of matrix deposition, however these studies continue to illustrate their capacities for stimulated biosynthesis in response to exogenous stimuli.

Using the immature bovine as the animal model allows for comparison of the behavior of the meniscus with the well characterized body of literature on immature bovine articular cartilage. However, one limitation of these studies is the use of a single animal model. The age of the animal strongly dictates the behavior of the cells in response to exogenous stimuli. Although we have not characterized the depth of penetration of the vascular supply, based on the age of the animal, the vascular supply is likely to extend into the middle zone of the meniscus, the origin of the tissue explants[20]. As the animal matures, the vascular zone is further limited to the outer one-third of the tissue. Based on the inner and outer comparisons in the current studies, future studies using a mature bovine model may provide information on the role of vascular access in directing fibrochondrocyte responses to external stimuli. Additionally, species differences exist and have been identified as potential explanations for differences between the current results and previously reported results, as mentioned in the earlier discussion sections.

To further characterize the effects of the growth factors on matrix deposition a more qualitative method could be used to look at the distribution within the agarose gels. In the current study, histological methods could also aid in interpreting differences in matrix quantity and synthesis seen in the rings and centers of the gels. The identification

of a viable cell distribution via live-dead staining with calcein AM-ethidium homodimer would aid in the analysis of the oscillatory compression data showing increased synthesis rates in the center of the gels. Matrix staining with safranin O and Mason's Trichrome would show the distribution of proteoglycans and collagen, respectively, in the matrix. More sensitive techniques such as immunofluorescent imaging as seen in Chapter 3 could be used to show distribution of matrix components. Also, Western blotting would provide a quantitative measure of matrix content. Additional studies that include the application of oscillatory compression coupled with growth factor supplementation throughout the duration of culture may allow for the development of a more homogeneous matrix.

As mentioned in the introduction, these studies focused only on the anabolic actions of cytokines. As the role of the meniscus in the pathogenesis of degenerative diseases becomes more clear, it will be important to understand the actions of catabolic cytokines on meniscus degeneration in a mechanically loaded environment. Work in our laboratory has shown release of glycosaminoglycans and collagen from the meniscus extracellular matrix in response to IL-1α treatment, a widely used model for studying processes involved in osteoarthritis[99], as well as intense staining for aggrecan degradation fragments in the inner region of the tissue. Under identical culture conditions, the profiles in release of cartilage matrix components were similar, but lagged the meniscus profiles by 2-4 days. Accordingly, meniscus explants also exhibited a more rapid loss of material properties than previously reported for cartilage explants. These findings suggest an increased susceptibility of the meniscus to cell-mediated degradation over that of cartilage, further supported by the clinical evidence of degenerative meniscal lesions

during the early onset of osteoarthritis in patients and the asymptomatic population[91,92]. Additionally, Kurz *et al.* have demonstrated upregulation of MMP-3 expression and the release of aggrecanase cleaved aggrecan fragments in to the media, suggestive of similar pathways of degradation in cartilage and fibrocartilage[163]. These studies identify events that are part of the catabolic cascade in disease progression within the meniscus. Therapeutic approaches may target any one of these events, however with the inhibition of these catabolic events, there also needs to be a commencement of anabolic events to rebuild the lost matrix components, hopefully restoring functional tissue capacity. Therefore, the current studies have provided a baseline for the response of normal, healthy tissue that can be used as an end goal for restoration of "normal" anabolism following tissue insult.

These current findings also have broader implications on the targeting of therapeutics. Stimulation of matrix synthesis by either a given biomechanical or biochemical stimulus has been shown to depend on the matrix environment as well as the cell type. Future interventions suggested by the seemingly inherent susceptibility of the meniscus to osteoarthritic degradation may focus on early treatment of the meniscus to delay the onset of degeneration. However, it is important to recognize the need for a thorough investigation of the potential effects of biochemical (cytokine treatment) or biomechanical (immobilization, passive motion, *etc.*) treatments. The current studies have shown differential effects of physiologically relevant stimuli on cells from tissues that share a common synovial joint environment. Therefore, consideration of the effects of a specific treatment on the target tissue as well as neighboring tissues will be necessary to ensure net positive effects on the overall health of the knee joint.

APPENDIX A

OSCILLATORY COMPRESSION DEVICE

A.1 Design

The oscillatory loading device presented in Chapter 5 and shown in Figure 19 was designed in our laboratory. The design of the polysulfone chambers is adapted from a previous version of Hunter's loading device[116]. The new design incorporates a few important ideas from the previous design: [1] including slots in the tops and bottoms allowing for the use of these chambers in static compression studies as well as oscillatory compression studies, [2] presence of the medium ports allowing for media feeds without disassembling the chambers, and [3] being able to run multiple samples in once chamber. This new design is more robust in sample capacity than the previous design, compressing up to 32 samples in a single device.

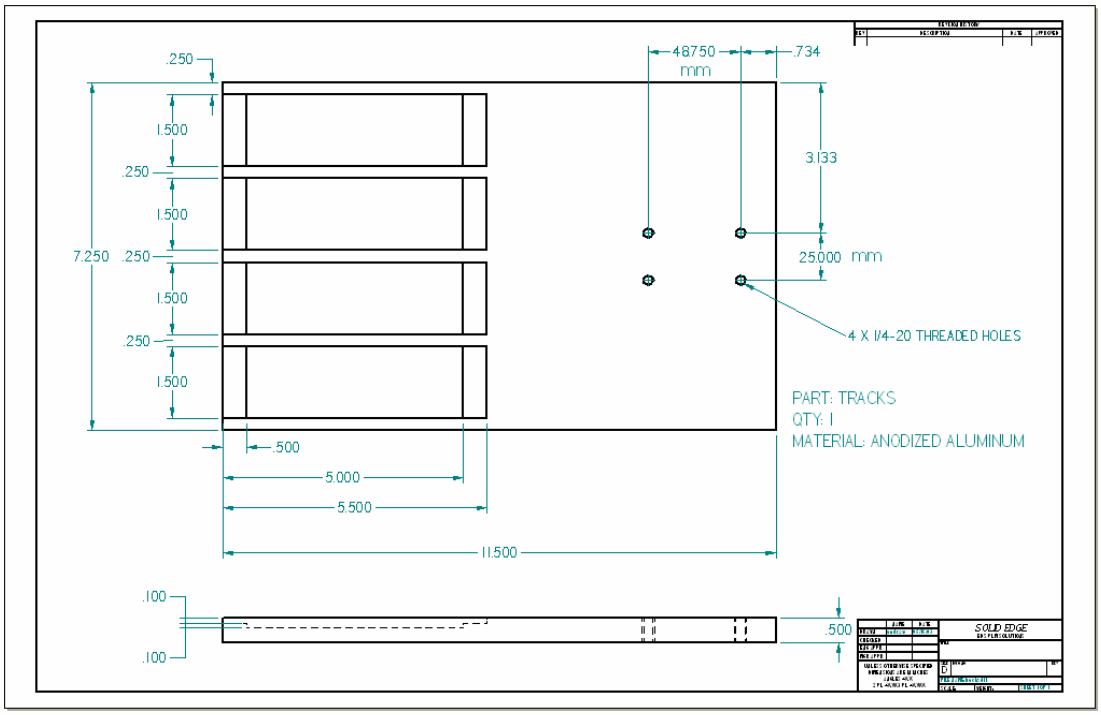

Figure A.1: Base plate of oscillatory loading device. This anodized aluminum type 6061 base plate anchors the bracket at the four holes shown on the right side of the plate. The polysulfone compression chambers slide into the tracks shown on the left side of the plate.

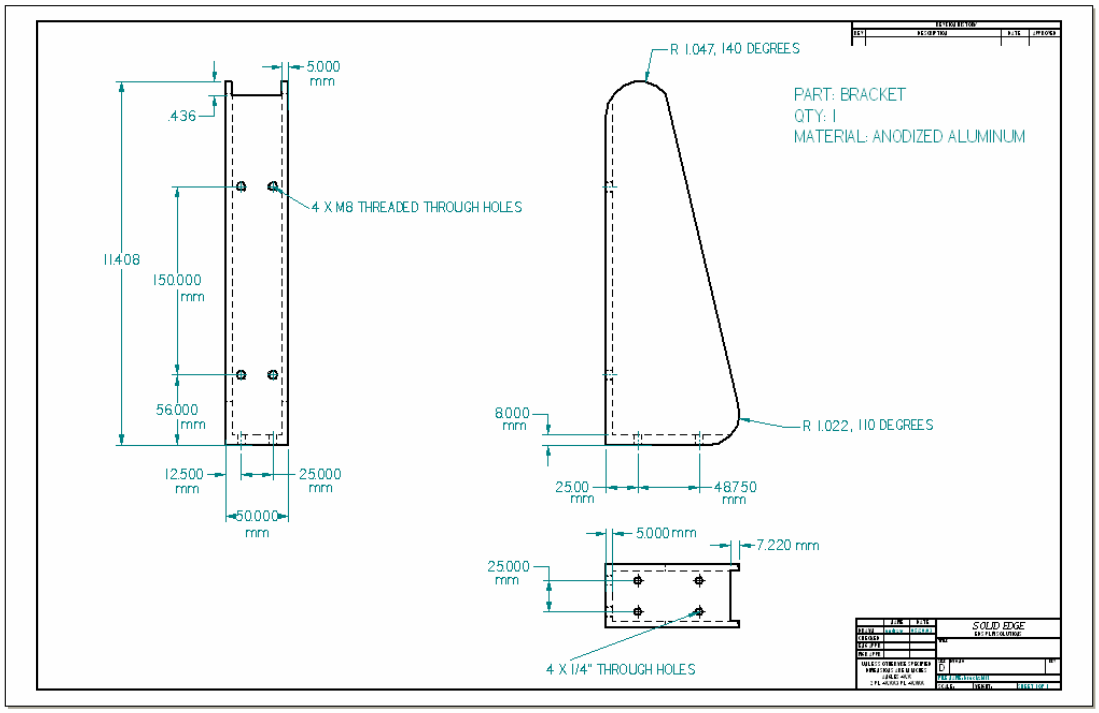

Figure A.2: Bracket of the oscillatory loading device. This anodized aluminum type 6061 bracket supports the vertically mounted 404XR linear table, by anchoring it to the base plate.

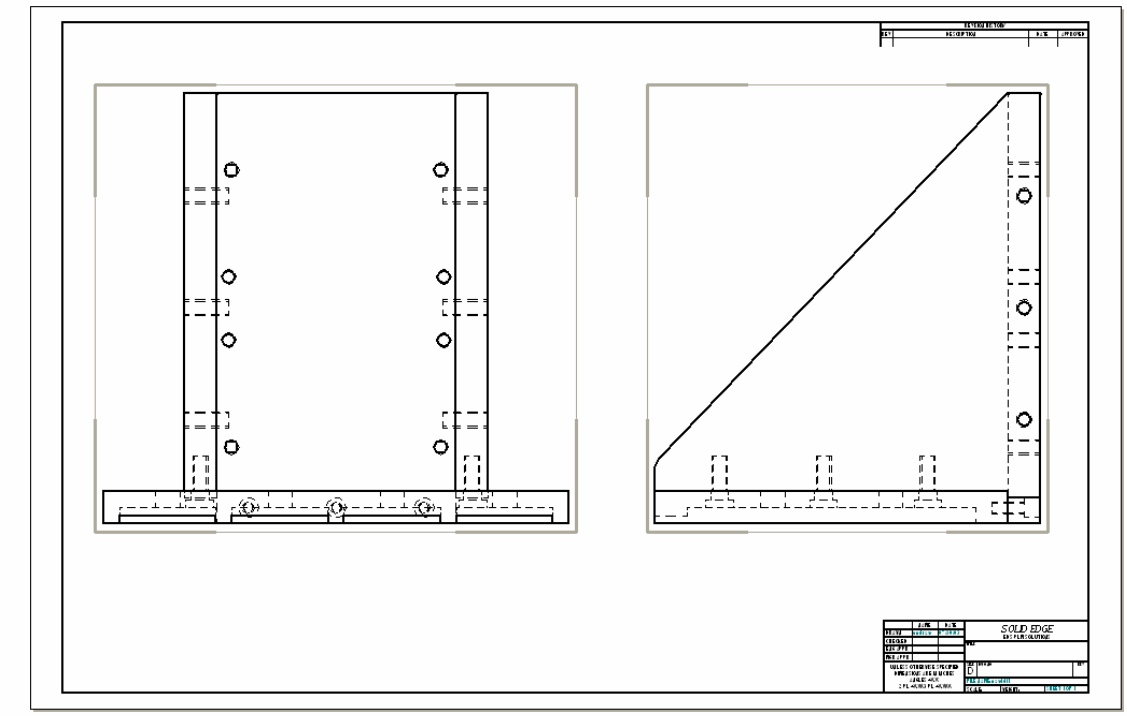

Figure A.3: L-bracket of the oscillatory loading device. This anodized aluminum type 6061 part is an assembly of the next three drawings (back, triangles [2], and bottom). The back face of the L-bracket affixes directly to the sliders of the 404XR. The polysulfone compression chamber tops attach to the L-bracket bottom.

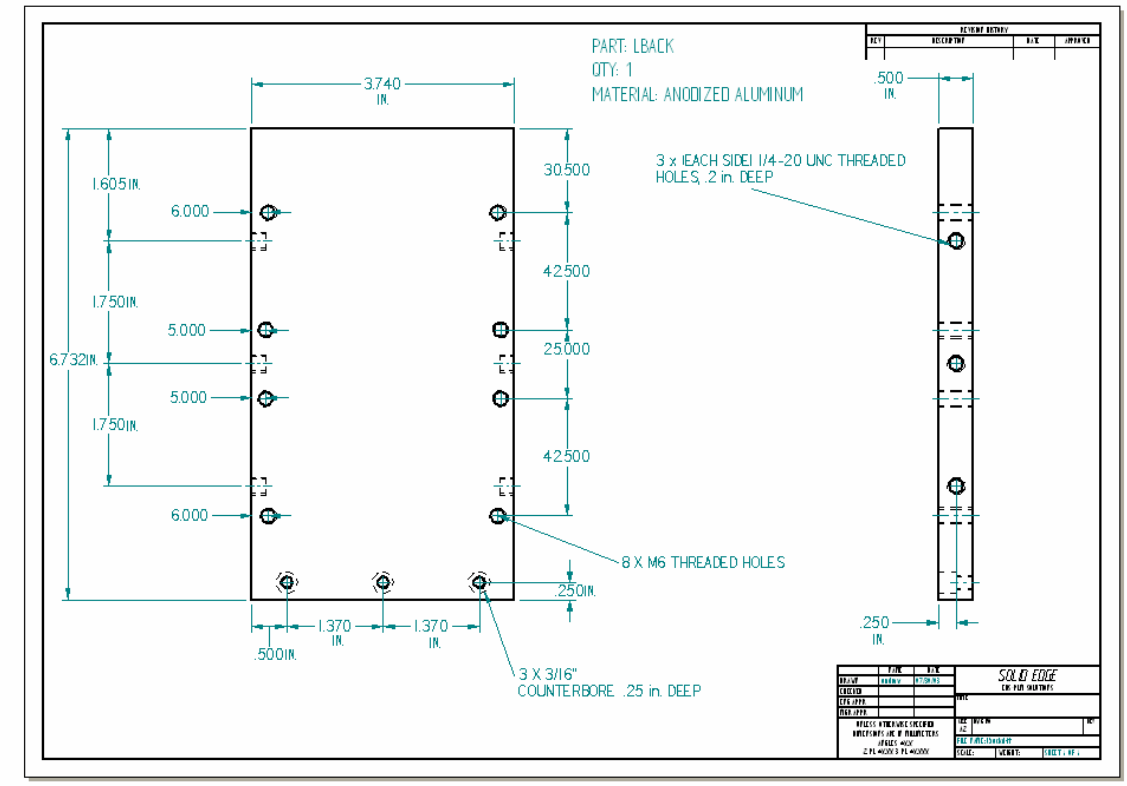

Figure A.4: Part 1 of 3 of L-bracket. This anodized aluminum type 6061 part is the back of the L-bracket and directly attaches the L-bracket to the sliders of the 404XR.

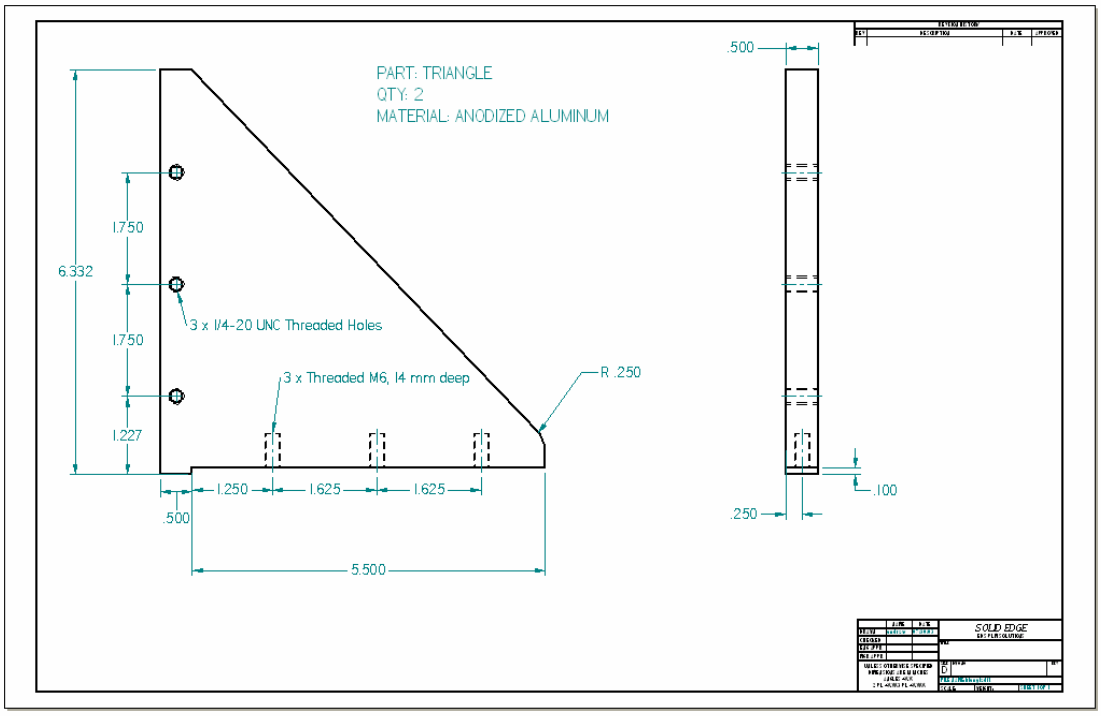

Figure A.5: Part 2 of 3 of the L-bracket. Two of these anodized aluminum type 6061 parts affix to the sides of the L-bracket and aid in the rigidity of the bracket.

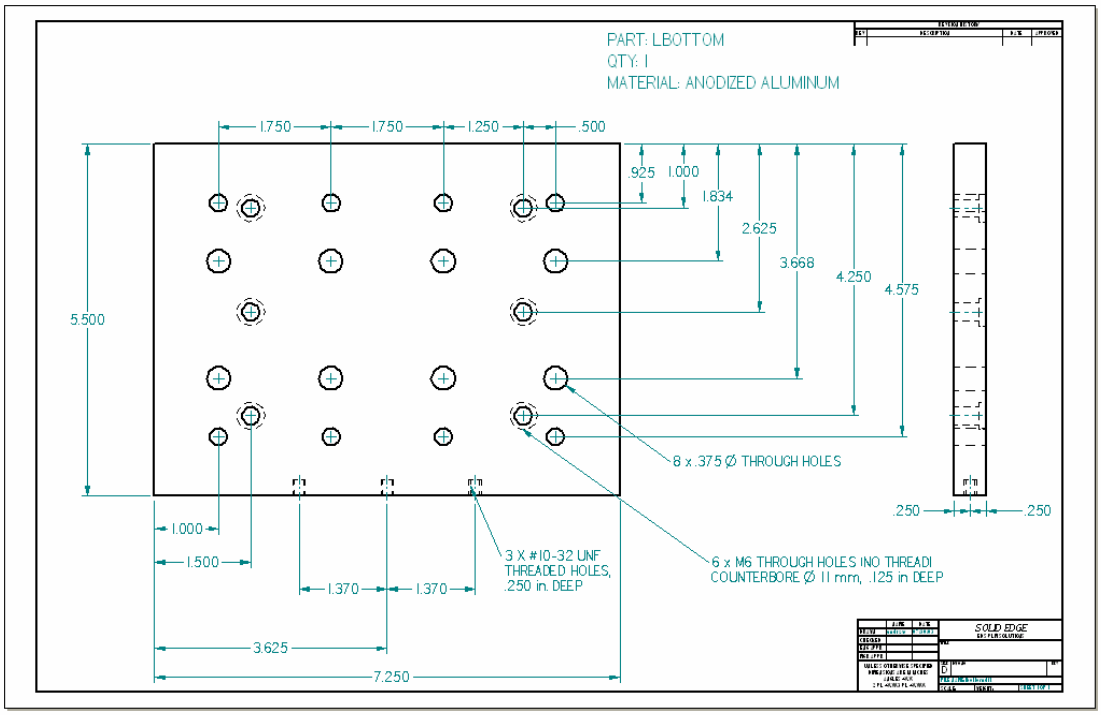

Figure A.6: Part 3 of 3 of the L-bracket. This anodized aluminum type 6061 part is the bottom of the L-bracket assembly. The compression chamber tops are attached directly to this part with bolts that extend through the 0.375" ∅ through holes and screw into the chamber tops.

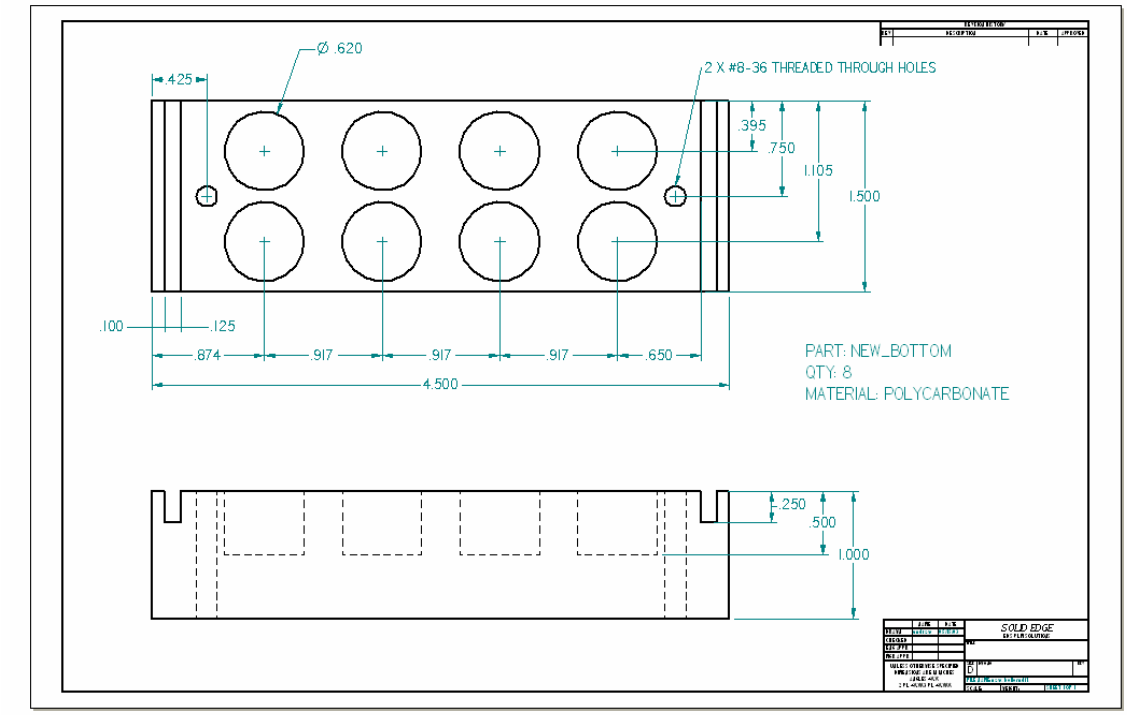

Figure A.7: Compression chamber bottom. This polysulfone chamber can hold eight individual samples. Stainless steel spacers machined to specific heights sit in the slots on either end of the chamber, offsetting the chamber tops and allowing for use in static compression studies as well as oscillatory compression studies.

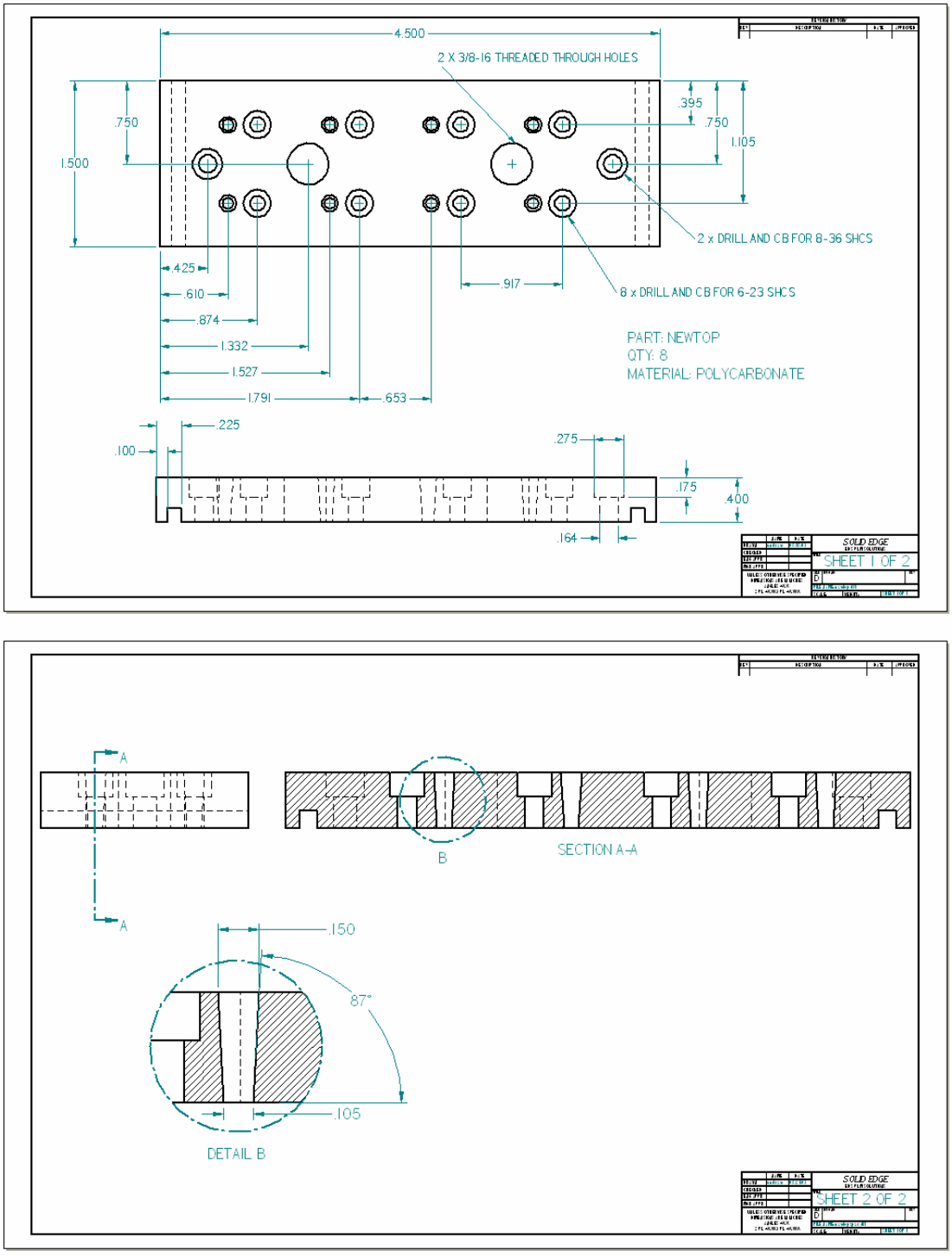

Figure A.8: Compression chamber top. Eight stainless steel platens are attached to the chamber top such that they align concentrically with compression chamber bottom wells (top). Tops have matching slots on either end for stainless steel static spacers used in static compression studies. Additionally, (bottom) medium ports allow for aspiration of old medium and addition of fresh medium while maintaining compression of the samples.

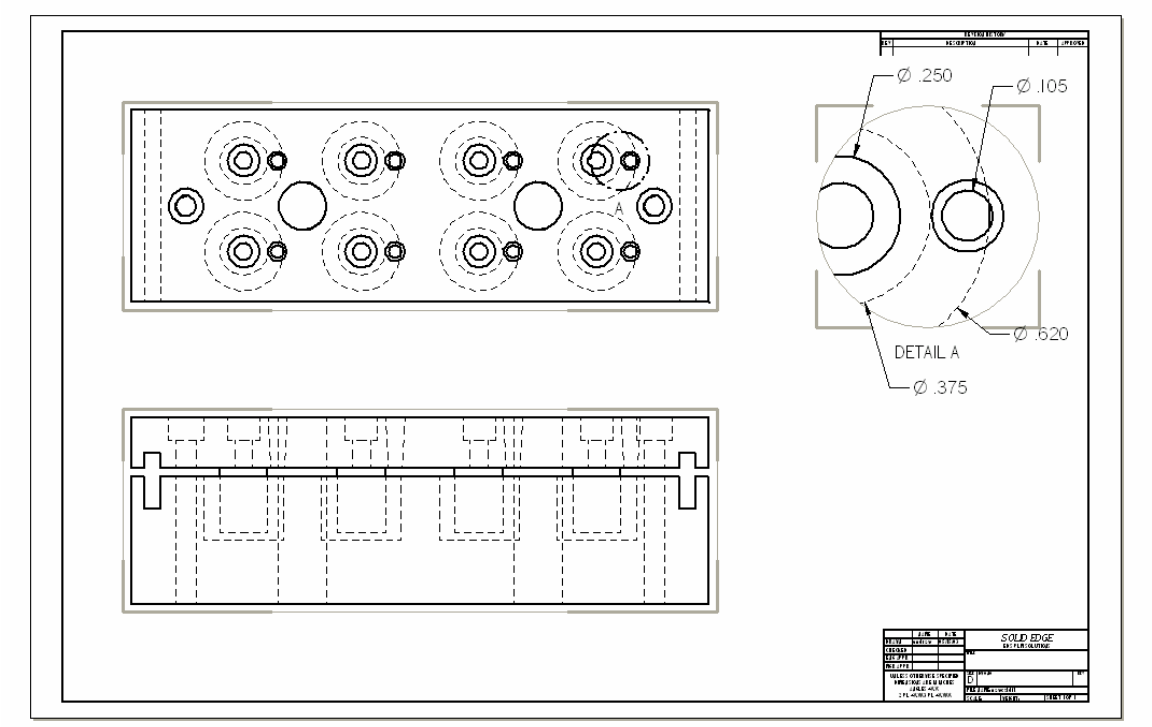

Figure A.9: Compression chamber assembly. Stainless steel spacers can be placed in the slots on far ends of the assembly to impart static compression. The inset shows a top view looking down on the media port that illustrates the clearance between the 0.375" ∅ stainless steel platen and the inside of the 0.620" ∅ well.

A.2 USEFUL COMMENTS IN THE GALIL WSDK SOFTWARE

General Commands

MO	Motor off	Disables the motor. You should hear a click in the control box after entering this command.
SH	Servo here	Enables the motor. You should hear a click in the control box and a hum from the enabled motor after entering this command.
BG	Begin	Begins a move. Typically used with the PA command
ST	Stop	Stops the current executable motion
XQ	Execute	Executes a program that has been downloaded to the controller

Interrogation Commands

These commands can be typed in to find out information about the system. The response will appear in the bottom left window. Commands can be typed in during motion.

TP	Tell position	Tells the current position of the platform from the most recently defined zero point.
TE	Tell error	Tells the error between the desired position and the actual position of the platform

Tuning Parameters

These commands are followed by a numerical string to set the values. To find out the most recent value of these parameters, type in the code followed by '?' (example: KP?).

KP	Proportional gain	P of the PID, currently = 3
KI	Integral gain	I of the PID, currently = 0
KD	Derivative gain	D of the PID, currently = 40
OF	Voltage offset	Sets an offset to counteract gravity, currently = 0.06

Binary Parameters

These commands are important in the smooth running and are either values of 1 (ON/enabled) or 0 (OFF/disabled). These commands are followed by a 1 or 0 to set the values. To find out the most recent value of these parameters, type in the code followed by '?'.

OE	Off-on-Error	When enabled will disable the motor when an error threshold has been exceeded. Currently set as 1 in the CIRCLES program.
DV	Dual Velocity Loop	When disabled, the PID parameters all work on the same feedback loop (the linear encoder). Currently set as 0 for smooth running of the CIRCLES program.

Motion Commands

These commands set values necessary for motion. These commands are followed by a numerical string to set the values.

DP	Define position	Defines the current position as whatever number of counts you specify. Usually used to specify the zero point
PA	Position absolute	Commands the position of the platform to be at xxx number of counts relative to the zero point
SP	Speed	Specifies the speed of movement in counts/sec
AC	Acceleration	Specifies the acceleration of movement in counts/sec^2
DC	Deceleration	Specifies the deceleration of movement in counts/sec^2

A.3 PROTOCOL FOR SETTING UP AND OSCILLATORY LOADING STUDY

1. Check the system out before you load your samples
    a. If the control box and computer are OFF, power them both ON.
    b. Open up WSDK
    c. Open up the terminal in the top toolbar
2. Type in MO to disable the motor
3. Check the PID parameters by typing in KP?, KI?, and KD?
4. Set P/I/D parameters to 3/0/30 by typing KP3, KI0, KD30 [BLUE frame]

    2.5/0/18 by typing KP2.5, KI0, KD18 [GOLD frame]
5. Set the voltage offset to -0.055 by typing OF-0.055 [BLUE frame]

    0.055 by typing OF0.055 [GOLD frame]
6. Set the mode to single loop by typing DV0
7. In the right window (program window), open the program for the oscillatory compression = CIRCLES_FORE_ORIGINAL_SLOW
8. Download the program to the controller by hitting the download button
9. Type in XQ to execute the program to check for stability
    a. If stable, type in ST to stop the execution. The motor will still be enabled at this point until you type in MO. Continue to step 10.
    b. If unstable, hit the kill switch and exit out of all software. Cycle the power on the control box and start from the beginning.
10. Raise the platform to a location that is reasonable for you to get your cassettes loaded.
    a. Do this by setting the speed to 3000 counts/sec by typing SP3000
    b. Set acceleration and deceleration to 25600 counts/sec^2 by typing AC25600 and DC25600
    c. Also, disable the Off-on-error command that was in the oscillatory compression program by typing in OE0

d. Remember ↑- and ↓+ AND 1 count = 0.1 μm. I usually raise the platform by 5000 or 10000 counts at a time

e. Define current position as 0 by typing DP0

f. Raise platform by typing in PAxxx and then BG

11. Disable the motor by typing MO

12. Load your cassettes in the hood

13. Take the rig out of the incubator and place cassettes in the RIG

14. Manually push down on the platform until it is in contact with the tops of the cassettes. Simultaneously, type in DP0 to reset the new zero position.

    This next part is optional or you can skip to step 24:

15. Enable the motor by typing SH

16. Raise platform by typing PA-40000 and then BG so that you can get your cassettes out

17. Return platform to the zero point by typing PA0 and then BG. Check position by typing in TP because it often doesn't make it all the way to zero. Retype PA0 to get the platform as close to zero as possible.

18. Download the CIRCLES program

19. Type in XQ to check for stability

20. Type in ST. Also, disable the Off-on-error command that was in the oscillatory compression program by typing in OE0

21. Raise platform by typing PA-40000 and then BG so that you can get your cassettes in the RIG

22. Place cassettes in the RIG and return the platform close to the tops of the cassettes (within 1000 counts). Do this gradually until you get to -1000 counts.

23. Disable the motor by typing MO.

24. Lightly screw in the large bolts (5/16" allen wrench) such that they are attached to the cassette tops

25. Unscrew the small alignment screws (M4 allen wrench) and take them out of the assembly

26. Fully tighten the large bolts

27. Enable the motor by typing SH

28. Slide the spacers out from the assembly

    a. If the spacers don't slide out so easily, raise the platform by 1000 counts to release the pressure from the assembly.

    b. Do not raise the platform beyond 2000 counts! This corresponds to 200 μm from the 10% static offset. Your samples my float out from under the platens if they are raised too high!

29. Return the platform to the zero point by typing PA0 and then BG.

30. Check that the tuning parameters and the binary parameters (from Useful Commands) are correct.

31. Download the CIRCLES program

32. Type in XQ to start the loading.

A.4 OSCILLATORY COMPRESSION PROGRAM

This program is written in the Galil WSDK software and programming language. The program utilizes the circle (CR) function to impart a sinusoidal motion. The commands that control the specific motion are: vector speed (VS) and circle (CR). Vector speed is an integer that is a product of: $1.024*(2\pi R f)$. R is the radius in linear encoder counts (1 count = 0.1 μm) of the circle. In the sinusoidal application, R represents the amplitude of the sine wave. f is the frequency in Hz. The value of 1.024 represents necessary compensation for a lag in time between the controller and the command, as stated in the Galil literature. The circle command is composed of three arguments: CR R,θ,Δθ. R is the same value from the VS command. θ is where on the circle the motion will begin from in degrees. Δθ is the number of degrees around the

circle that the command will follow. In the program θ is set at -90° to begin at a 0 position, and Δθ is set at 360° to complete a single circle. The CR command exists in a jump (JP) loop, and therefore is commanded to continue until manually stopped.

Program

```
#CIRCLES_FORE_ORIG_SLOW

VMAN
SH

OE1
ER 1200

DP0
VA 680000
VD 680000
VS 5787
CR 900,-90,360
BGS

#L
CR 900,-90,360
#WAIT ; JP #WAIT ; _LM=0
JP#L

EN
```

APPENDIX B

LIST OF REAGENTS AND MATERIALS

Product	Vendor	Location
Collagen I Antibody	Abcam	Cambridge, MA
Collagen II Antibody	Abcam	Cambridge, MA
Collagen VI Antibody	Abcam	Cambridge, MA
e-aminocaproic acid	Acros Organic	Fairlawn, NJ
L-5-^{3}H-proline	American Radiolabeled Chemicals	St. Louis, MO
SYBR Green Master Mix	Applied Biosystems	Foster City, CA
Glass Electrophoresis Plates	Bio-Rad	Hercules, CA
T-75 Flasks	Corning	Corning, NY
0.22 mm Polyethersulfone Filter	Corning	Corning, NY
Aggrecan-G1 Antibody	Dr. John Sandy Shriner's Hospital	Tampa, FL
Proteinase K	EMD Chemicals	Gibbstown, NJ
b-Mercaptoethanol	EMD Chemicals	Gibbstown, NJ
24-well Plates	Falcon	Franklin Lakes, CA
48-well Plates	Falcon	Franklin Lakes, CA
Triton X-100	Fisher Scientific	Pittsburg, PA
Sodium Sulfate	Fisher Scientific	Pittsburg, PA
Fetal Bovine Serum	Hyclone	Logan, UT

Product	Vendor	Location
Bovine Fibrinogen	ICN Biomedical	Irvine, CA
Thrombin	ICN Biomedical	Irvine, CA
Ecolume	ICN Biomedical	Irvine, CA
High Glucose Dulbecco's Modified Eagle's Medium	Invitrogen	Carlsbad, CA
HEPES Buffer	Invitrogen	Carlsbad, CA
Non-essential Amino Acids	Invitrogen	Carlsbad, CA
Gentamicin	Invitrogen	Carlsbad, CA
Fungizone (Amphotericin B)	Invitrogen	Carlsbad, CA
Phosphate Buffered Saline	Invitrogen	Carlsbad, CA
Collagenase Type II	Invitrogen	Carlsbad, CA
Trypsin	Invitrogen	Carlsbad, CA
Neomycin	Invitrogen	Carlsbad, CA
Kanamycin Sulfate	Invitrogen	Carlsbad, CA
PSN	Invitrogen	Carlsbad, CA
Forward and Reverse Primers	Invitrogen	Carlsbad, CA
Trizol Reagent	Invitrogen	Carlsbad, CA
Polysulfone	JM Machining	Lawrenceville, GA
p-DAB	JT Baker	Phillipsburg, NJ
Chloramine T	Mallinckrodt	Paris, KY
Red FDA Rubber	McMaster Carr	Atlanta, GA
4 mm Biopsy Punch	Miltex	York, PA
6 mm Biopsy Punch	Miltex	York, PA
Labeled Goat a-Rabbit IgG Antibody	Molecular Probes	Eugene, OR
Labeled Goat a-Mouse IgG Antibody	Molecular Probes	Eugene, OR
Phalloidin	Molecular Probes	Eugene, OR

Product	Vendor	Location
^{35}S-sodium sulfate	MP Biomedicals	Irvine, CA
rhbFGF	PeproTech	Rocky Hill, NJ
rhIGF-I	PeproTech	Rocky Hill, NJ
rhPDGF-AB	PeproTech	Rocky Hill, NJ
rhTGF-b1	PeproTech	Rocky Hill, NJ
Promega Reverse Transcription Kit	Promega	Madison, WI
Qiagen Rneasy Mini Kit	Qiagen	Valencia, CA
Qiagen Qiashredders	Qiagen	Valencia, CA
Calf Stifle Joints	Research 87	Marlborough, MA
L-proline	Sigma	St. Louis, MO
L-ascorbic acid	Sigma	St. Louis, MO
Bovine Serum Albumin	Sigma	St. Louis, MO
Agarose	Sigma	St. Louis, MO
Ammonium Acetate	Sigma	St. Louis, MO
Agarase	Sigma	St. Louis, MO
1,9-Dimethylmethlyene Blue	Sigma	St. Louis, MO
Hoechst 33258 Dye	Sigma	St. Louis, MO
Chondroitin Sulfate	Sigma	St. Louis, MO
Calf Thymus DNA	Sigma	St. Louis, MO
Hydroxyproline	Sigma	St. Louis, MO
Goat Serum	Sigma	St. Louis, MO
Rabbit Serum	Sigma	St. Louis, MO
No. 12 Razor Blades	VWR Scientific	West Chester, PA
#22 Scalpel Blade	VWR Scientific	West Chester, PA

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VITA

Stacy Marie Imler was born in Seoul, South Korea in 1977 and soon after landed in New York City (along with the pearl cream) to be adopted by Frederick Spencer and Sheila Ellen Imler. After moving to Hazlet, New Jersey at the age of 8 and believing that babies came from airplanes (after seeing her adopted sister, Robyn Dyan, arrive at the airport), she attended Lehigh University in Bethlehem, PA. In May of 1998, she graduated Summa Cum Laude with a Bachelor's of Science Degree in Mechanical Engineering with Division I Varsity letters in Cross Country and Track & Field. Stacy had a summer internship at Lucent Technologies in Whippany, NJ prior to her senior year. She also spent the summer after graduation in Phoenix, AZ as an intern at AlliedSignal. She arrived in Atlanta, GA in September 1998, where she began her graduate career at the Georgia Institute of Technology in Mechanical Engineering. She was awarded a Clare Booth Luce Graduate Fellowship as well as a National Science Foundation Graduate Fellowship. She received her Master of Science Degree in May of 2001 in Mechanical Engineering and Doctor of Philosophy Degree in August of 2005. During her tenure at Georgia Tech, she was an avid intramural participant, involved in the ultimate Frisbee community of Atlanta, a finisher of the 2001 New York City Marathon, a 2-time Pi Mile winner, a 2-time Whopper Queen, and a first-time thespian in the 2005 campus production of The Vagina Monologues. She also enjoys spending time with her cat, Hansel, and drinking beer with her friends. She plans on obtaining an industrial job after finishing the preparation and submission of several manuscripts.